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(57) Abstract

Isolated DNA encoding the catalytic subunit of a eukaryotic telomerase holoenzyme, such as the catalytic subunit of a yeast or human telomerase holoenzyme; the RNA transcript, which is expressed in primary human tumors, cancer cell lines and telomerase–positive tissues; and the encoded catalytic subunit protein. Methods of assessing cells for malignancy or an increased likelihood of progression to malignancy and methods of diagnosing or aiding in the diagnosis of development of malignancy in an individual are also described.

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TELOMERASE CATALYTIC SUBUNIT GENE AND ENCODED PROTEIN

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RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/038,750, filed February 20, 1997, entitled "Identification of a Protein Subunit of the Yeast Telomerase Holoenzyme" by Christopher M. Counter, Matthew Meyerson and Robert A. Weinberg; of U.S. Provisional Application No. 60/047,151, filed May 20, 1997, entitled "Human Telomerase Catalytic Subunit" by Christopher M. Counter and Matthew Meyerson; of U.S. Provisional Application No. 60/054,549, filed August 1, 1997, entitled "hEST2, the Putative Human Telomerase Catalytic Subunit Gene Is Up-Regulated in Tumor Cells and During Immortalization" by Matthew Meyerson, Christopher M. Counter and Robert A. Weinberg; of U.S. Provisional

Application No. 60/055,762, filed August 14, 1997, entitled "Human Telomerase Catalytic Subunit Gene and Uses Therefor" by Matthew Meyerson, Christopher M. Counter and Robert A. Weinberg; and of U.S. Provisional Application No. 60/064,322, filed October 30, 1997, entitled "Human Telomerase Catalytic Subunit Gene and Uses Therefor", by Matthew Meyerson, Christopher M. Counter and Robert A. Weinberg. The entire teachings of these five referenced applications are expressly incorporated herein by reference.

BACKGROUND OF THE INVENTION

In most eukaryotes, the telomere ends of linear chromosomes are replicated by the ribonucleoprotein enzyme telomerase (Blackburn, E.H. *Annu. Rev. Biochem. 53*, 163-94 (1984); Zakian, V.A., *Science 270*, 1601-7 (1995); Greider, C.W. & Blackburn, E.H., *Cell 43*, 405-13 (1985)). The RNA subunit of this enzyme has now been described in multiple species (Blasco, M.A. *et al.*, *Science 269*, 1267-70 (1995); Feng, J., *et al.*, *Science 269*, 1236-41 (1995); Greider, C.W. & Blackburn, E.H., *Nature 337*, 331-7 (1989); Lingner, J. *et al.*, *Genes Dev. 8*, 1984-98 (1994); McEachern, M.J. & Blackburn, E.H., *Nature 376*, 403-9 (1995); Melek, M. *et al.*, *Mol. Cell Biol. 14*, 7827-38 (1994); Shippen-Lentz, D. & Blackburn, E.H., *Science 247*, 546-52 (1990); Singer, M.S. & Gottschling, D.E., *Science 266*, 404-9 (1994)). Candidate protein subunits have been isolated in the ciliate *Tetrahymena* (Collins, K.*et al.*, *Cell 81*, 677-86 (1995) but not, however, in any genetically tractable organism.

Telomerase is repressed in normal human somatic cells but is re-activated during tumor progression. This re-

activation is not reflected by changes in the levels of previously cloned genes encoding telomerase subunits.

SUMMARY OF THE INVENTION

Described herein are genes which are required for telomerase enzymatic activity and the respective encoded messenger RNAs (transcripts) and proteins. As described below, the present invention relates to DNA which is involved in telomere length regulation in eukaryotes and proteins which are physically associated with the respective active telomerase enzyme and, thus, are each a component of the respective telomerase holoenzyme. In one embodiment, the gene, RNA transcript and encoded protein are a yeast gene and its encoded RNA transcript and protein. In a second embodiment, the gene, RNA transcript and encoded protein are human. Also described herein is a human cDNA which encodes the human telomerase catalytic protein subunit.

The yeast telomerase gene is present on chromosome XII and its disruption alters telomere maintenance, as demonstrated by the decrease in telomere length in transposon-insertion mutants. The sequence of the gene, referred to herein as *EST2* (SEQ ID NO.: 1), the deduced amino acid sequence of the encoded *Est2* protein, also referred to as Est2p, (SEQ ID NO.: 2) and the hEST2 RNA transcript (SEQ ID NO.: 36) are provided. The DNA has been shown to be essential for telomerase activity in yeast and the encoded protein has been shown, by the methods described herein, to be physically associated with telomerase and a constituent of active telomerase complex in yeast. The yeast telomerase DNA *EST2* is a subject of

the present invention; this includes isolated DNA comprising DNA selected from the following: DNA of SEQ ID NO.: 1; DNA which is the complement of SEQ ID NO.: 1, DNA which hybridizes to DNA of SEQ ID NO.: 1 or a complement thereof; DNA which localizes to yeast chromosome XII; and DNA which encodes the amino acid sequence of SEQ ID NO.: 2. Isolated yeast Est2p is also the subject of this invention. This includes isolated Est2p of SEQ ID NO.: 2 and Est 2p encoded by EST2 DNA as defined herein.

The gene described herein is useful to identify genes encoding telomerase proteins in other eukaryotes, particularly in vertebrates, including mammals and especially humans. All or a portion of the gene described can be used. The encoded portion can be used to produce antibodies (monoclonal or polyclonal) which bind the *Est2* protein and can, in turn, be used to identify corresponding proteins (proteins physically associated with telomerase enzymatic activity) in other eukaryotes.

Also described herein is a human cDNA, which was originally named *hEST2* and has been renamed *hTERT*, that encodes the human telomerase catalytic protein subunit, the encoded *hEST2* RNA and the encoded hEST2 protein. The human DNA described herein is a human homologue of yeast and ciliate genes which encode telomerase catalytic subunits; it shares significant sequence similarity with the telomerase catalytic subunit genes, yeast *EST2* and Euplotes p123. *hEST2* RNA expression reflects telomerase activity. The RNA transcript is expressed in primary human tumors, cancer cell lines and telomerase-positive tissues, but is undetectable in telomerase-negative cell lines and differentiated telomerase-negative tissues. *hEST2* message is absent in pre-crisis, telomerase-negative transformed cells, but is readily detectable in post-crisis,

telomerase-positive immortalized cells. Taken together, these observations are evidence that the induction of *hEST2* mRNA expression is required for the telomerase activation that occurs during cellular immortalization and tumor progression.

The encoded human protein comprises six of the seven conserved sequence motifs which define the polymerase domains of members of the reverse transcriptase family and also includes the invariant aspartic acid residues required for telomerase enzymatic activity. Although hEST2 protein comprises such motifs which define polymerase domains, beyond these it shows no sequence similarity with reverse transcriptases. It is more closely related to the telomerase catalytic subunits of yeast and ciliates than to other reverse transcriptases. In its domains that lie N-terminal to the polymerase domain, hEST2 shows clear relatedness to both p123 and Est2p. Many of the sequence identities in the N-termini of the three proteins are in a region just before motif 1. These sequences do not appear in reverse transcriptases or in other proteins and, thus, appear to be unique to telomerases. For example, these three include a unique motif, referred to as the telomerase motif; in hEST2 this motif extends from amino acid residue 556 to amino acid residue 565 of SEQ ID NO.: 3, with absolute invariant sequence extending from amino acid residue 560 to amino acid residue 565 of SEQ ID NO.: 3. Further, within the hEST2 domain that shares sequence similarity with reverse transcriptases, it is clear that hEST2 is more closely related to telomerase reverse transcriptases than to non-telomerase reverse transcriptases. The three telomerase catalytic subunits (hEST2, yeast EST2 and Euplotes p123) form a subgroup within the reverse transcriptase family.

The present invention relates to isolated DNA which encodes an RNA transcript which is expressed in primary human tumors, cancer cell line and telomerase-positive tissue. In one embodiment, the isolated DNA comprises *hEST2* DNA such as DNA comprising SEQ ID NO.: 35 (DNA comprising the nucleotide sequence of SEQ ID NO.: 35). Isolated DNA of the present invention encodes an RNA transcript which is not detectable in telomerase-negative cell lines or in differentiated telomerase-negative tissues. Further, isolated DNA of the present invention encodes an RNA transcript which is not detectable in pre-crisis, telomerase-negative transformed cells and is detectable in post-crisis, telomerase-positive immortalized cells. In a particular embodiment, the DNA comprises *hEST2* cDNA, such as SEQ ID NO.: 35 or DNA which hybridizes thereto or to a complement of *hEST2* cDNA (such as a complement of SEQ ID NO.: 35) under conditions of high stringency. A further subject of the present invention is an isolated RNA transcript encoded by isolated DNA of the present invention.

A method of altering telomerase function and, thus, of altering telomere shortening is also described. In the method, expression and/or function of the telomerase-associated protein is altered (enhanced or reduced), resulting in altered (enhanced or reduced) cell lifespan. Expression of the telomerase-associated protein is enhanced, for example, by introducing DNA encoding the protein into cells. Alternatively, telomerase-associated protein is introduced into cells. Expression of the telomerase-associated protein is reduced, for example, by introducing into cells an agent which inhibits production or function of the protein, an agent which destroys the expressed protein or a dominant negative form of the

protein. Therefore, the present invention also provides a method of increasing the lifespan of a cell or, alternatively, of reducing the lifespan of a cell, such as that of cancer cells or transformed cells. In the method of increasing lifespan of cells, telomerase activity is maintained or increased within the cells, such that telomeres are maintained and as the cells age, telomere shortening does not occur or occurs to a lesser extent than would otherwise be the case (if telomerase activity were not maintained or increased). Reduction of lifespan of cells, such as tumor cells, is accomplished by introducing into the cells an inhibitor of the telomerase protein subunit described herein.

A method of assessing cells or aiding in the assessment of cells for malignancy or an increased likelihood of progression to malignancy is also the subject of this invention. In the method, cells to be assessed are obtained from an individual (e.g., a human) in need of such an assessment. The cells are processed in such a manner that DNA, RNA or both in the cells are rendered available for annealing or hybridization with complementary polynucleotides or oligonucleotides (DNA or RNA), such as probes or primers, thereby producing processed cells. The processed cells are combined with DNA or RNA required for telomerase enzymatic activity (DNA or RNA encoding a protein required for telomerase enzymatic activity), a complement of the required DNA or RNA or a characteristic portion or fragment of the DNA or RNA, such as the telomerase motif or all or a portion of the 5' end of *hEST2* which is not shared with the yeast or Euplotes gene. If hybridization occurs, it is indicative of the presence of telomerase protein-encoding DNA or RNA in the cells and of activation of telomerase, which is also indicative of malignancy or an increased likelihood of progression to

malignancy. As described herein, the *hEST2* DNA encodes an RNA transcript which is expressed in primary tumors, cancer cell lines and telomerase - positive tissues and is readily detectable in post-crisis, telomerase-positive immortalized cells, but not detectable in telomerase-negative cell lines and differentiated telomerase-negative tissues. Detection of *hEST2* DNA and/or of the RNA transcript, thus, makes it possible to detect DNA which encodes a transcript required for telomerase activation that occurs during cellular immortalization and tumor progression and to detect the RNA transcript itself.

In an alternative embodiment, the invention is a method of diagnosing or aiding in the diagnosis of development of malignancy (cancer, tumor formation) in an individual, in which the occurrence (presence or absence) and/or quantity of a telomerase protein (e.g., hEST2 protein) in cells is assessed. In the method, cells are obtained from an individual in need of diagnosis of malignancy and processed, if necessary, in such a manner that proteins in the cell are available for detection, such as by binding with antibodies or antibody fragments. The processed cells are combined or contacted with antibodies that recognize (bind) the telomerase protein (e.g., antibodies that bind Est2p or hEST2 protein) under conditions appropriate for antibody binding to occur. Whether binding occurs is determined; optionally, the extent to which binding occurs can also be determined. If binding of anti-telomerase protein antibodies (e.g., anti-hEST2 protein antibodies) to a component of processed cells occurs, it is indicative of the presence of hEST2 protein in the cells and of malignancy or an increased likelihood of development or malignancy in the individual.

In one embodiment, the present invention is a method of reducing expression of *hEST2* RNA and hEST2 protein in

cells of an individual (e.g., a human or other mammal). The method comprises administering to the individual a drug selected from the group consisting of drugs which inhibit (directly or indirectly) or bind *hEST2* RNA and prevent or reduce production of hEST2 protein and drugs which inhibit hEST2 protein function or activity. The drug is administered in a therapeutically effective amount (an amount sufficient to have the desired effect of reducing expression of *hEST2* RNA and hEST2 protein) and under conditions appropriate for entry into cells, in which they have the desired effect.

In a further embodiment, the present invention is a method of treating cancer in an individual, in need of such treatment. In the method, a drug which inhibits or binds *hEST2* RNA (or DNA) and prevents or reduces production of hEST2 protein or a drug which inhibits hEST2 protein function or activity is administered to the individual under conditions appropriate for entry into cells and in a therapeutically effective amount (an amount sufficient to have the desired effect of inhibiting *hEST2* RNA and/or preventing or reducing production of hEST2 protein or inhibiting hEST2 protein function or activity), with the result that cancer in the individual is treated (reduced, reversed or prevented from advancing).

A further embodiment of the invention is a method of altering (increasing or reducing) lifespan of cells in culture or in an individual. In the method in which lifespan is increased, *hEST2* DNA is introduced into cells (e.g., in culture or in the individual), in which the *hEST2* RNA transcript and encoded protein are produced in sufficient quantity to increase the lifespan of cells in the cultured cells or the individual. In the method in which lifespan is decreased, hEST2 protein function,

activity or production is reduced (partially or totally). This is done, for example, by introducing into cells in culture or administering to an individual in whom cell lifespan is to be decreased a drug which inhibits or binds *hEST2* RNA and prevents or reduces production of hEST2 protein; a drug which inhibits hEST2 protein function, activity or production; or a drug which inhibits or binds *hEST2* DNA and prevents or reduces production of *hEST2* RNA transcripts. The drug is administered to an individual under conditions appropriate for entry into cells in sufficient quantity to have the desired effect (in sufficient quantity to decrease lifespan of the cells by reducing hEST2 protein function, activity or production).

In the above-described embodiments, the drug can be, for example, a small organic molecule, an enzyme which degrades hEST2 protein, an enzyme inhibitor (e.g., a telomerase or hEST2 enzyme inhibitor), an hEST2 transcriptional regulator; an antisense molecule or a dominant negative form of hEST2 protein.

Also described herein is a mutant yeast strain, referred to as DN, which carries an inactive chromosomal rad52Δ allele. The RAD 52 coding sequence of a haploid strain of genotype *MATa leu 2-3, 112 lys2-201 trp1-1 ura3-52 his3-200* was replaced with a selectable marker (e.g., the *HIS3* gene), to generate strain DN. Mutant yeast strain DNR, in which the absence of *RAD52* was complemented by introducing the plasmid *pYPCR+*[35], which encodes the *RAD52* gene under it own promoter and the selectable marker *URA3*, is also described. Also described are mutant yeast strain *DNRtlcΔ*, in which the TLC1 gene in DNR cells was replaced with the LEU2 selectable marker gene and Y0035, in which

the Est2 gene has been mutated with the addition of a TN3 LEU2 transposon in the DNR genetic background.

The mutant yeast strain described herein requires telomerase activity for viability. It is useful to carry out a rapid and automatable biological assay for telomerase inhibitors and, thus, for inhibitors of telomere biosynthesis. The assay is also the subject of the present invention. The mutant yeast strain is also useful to identify agents which are recombination inhibitors. In the mutant yeast strain, referred to as DN, the *RAD52* recombination gene has been replaced by a *HIS3* gene. As described herein, disruption of the *TLC1* telomerase RNA subunit gene or the *MIT1* putative telomerase protein subunit gene in the *rad52* mutants leads to a delayed cell death. Yeast lacking both recombination activity and telomerase activity die after about 20 to 60 cell generations. Telomerase activity can be disrupted by enzyme inhibitors, as well as by genetic inactivation. Thus, for a telomerase inhibitor screen, test compounds are added, at different concentrations, to the DNA mutant strain (the test strain) and to a wild-type (control) strain. Periodically, such as each day, cultures of the two strains are diluted (e.g., 1:1000). Compounds that kill the yeast instantly or that kill the DNA and wild-type yeast with identical delays, are eliminated. Compounds that kill the test yeast strain significantly before the control yeast strain are telomerase inhibitors. This biological assay has several advantages over available methods. It is fast, automatable, and can be performed easily in large quantities. Because it is a biological assay, it reflects the physiological function of the drugs. Furthermore, it allows testing of biochemical and uptake parameters in a single step. Finally, this assay also

excludes inhibitors of other metabolic enzymes, such as RNA and DNA polymerases and is specific for telomerase inhibitors.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B present the nucleic acid sequence of the *EST2* gene (SEQ ID NO.: 1) and the predicted amino acid sequence of the Est2 protein (SEQ ID NO. 2).

Figure 2 shows alignment of the predicted amino acid sequence of hEST2, also referred to as *hTERT*, (SEQ ID NO.: 3) with the yeast Est2p (SEQ ID NO.: 2) and Euplotes p123 (SEQ ID NO.: 4) homologues. Amino residues within shaded blocks are identical between at least two proteins. Identical amino acids within the reverse transcriptase (RT) motifs (Poch *et al.*, *EMBO J. 8*:3867-3874 (1989); Xiong and Eickbush, *EMBO J. 9*:3353-3362 (1990)) are in black boxes, an example of a telomerase-specific motif in an outlined gray box, and all other identical amino acids in gray boxes. RT motifs are extended in some cases to include other adjacent invariant or conserved amino acids. The sequence of the expressed sequence tag AA281296 is underlined.

Figures 3A-3F show the alignment of RT motifs 1 to 6 of telomerase subunits hEST2 (SEQ ID NO.: 5-10), p123 (SEQ ID NO.: 11-16) and Est2p (SEQ ID NO.: 17-22) with S. cerevisiae group II intron-encoded RTs a2-Sc (SEQ ID NO.: 23-28) and a1-Sc (SEQ ID NO.: 29-34). The consensus sequence of each RT motif is shown (h: hydrophobic residues, p: small polar residues, c: charged residues). Amino acids that are invariant among telomerases and the RT consensus are boxed in black, while those that are invariant among telomerases and similar to the RT consensus are boxed in gray. Open boxes identify highly conserved

residues unique to either telomerases or to non-telomerase RTs. Asterisks denote amino acids essential for polymerase catalytic function.

Figure 4 is an ideogram of human chromosome 5p showing linkage of *hEST2* to sequence-tagged site (STS) markers WI-9907 and D5S417 determined by radiation hybrid (RH) mapping employing the Genebridge 4 RH panel (GB) (1 cR=270 kb). The higher resolution Stanford G3 RH panel (G3) placed *hEST2* close to marker D5S678 (1 cR=30 kb). The calculated distance between *hEST2* and these STS markers is in centiRays (cR) with LOD scores in parentheses.

Figures 5A-5B show the nucleotide sequence of *hEST2* cDNA in which the start codon ATG is underlined (SEQ ID NO.: 35).

Figure 6 is the amino acid sequence of hEST2 (SEQ ID NO.: 3).

Figures 7A, 7B and 7C are the nucleotide sequence of partially or alternatively spliced hEST2 message RNA (SEQ ID NO.: 36).

Figures 8A-8F show the expression of hEST2 in normal human tissues and cancer cell lines. Duplicate RNA blots were probed with an hEST2 probe (top panels) or with a β-actin probe as internal control (bottom panels). The 4.4 kb, 6 kb, and 9.5 kb transcripts are hEST2-specific.

Figures 9A-9D shows hEST2 expression in primary human tumors. Rnase protection assays are shown for hEST2 and β-actin controls. Sizes of the full-length and protected bands are indicated. Shown are the HL60 leukemia cell line (control), normal breast tissue, 2 primary breast tumors, the MCF7 and T47D breast cancer cell lines, and normal and primary tumor tissues from the testis, colon, and ovary. The doublet seen protected by the hEST2 probe is invariant, and may be a result of probe secondary structure.

DETAILED DESCRIPTION OF THE INVENTION

The linear chromosomes of eukaryotic cells offer the biological advantages of rapid recombination, assortment, and genetic diversification. However, linear DNA is inherently more unstable than circular forms. To address this difficulty, the eukaryotic chromosome has evolved to include a DNA-protein structure, the telomere, which caps chromosome ends and protects them from degradation and end-to-end fusion (Blackburn, *Ann. Rev. Biochem. 53:*163-194 (1984); Zakian, *Science 270:*1601-1607 (1995)).

The DNA component of telomeres consists of tandem repeats of guanine-rich sequences that are essential for telomere function (Blackburn, *Ann. Rev. Biochem. 53:*163-194 (1984), Blackburn, *Nature 350:569-573* (1991); Zakian, *Science 270:*1601-1607 (1995)). These repeats are replicated by conventional DNA polymerases and by a specialized enzyme, telomerase (Greider, In *Telomeres*, eds. Blackburn and Greider, Cold Spring Harbor Laboratory Press, pp. 35-68 (1995)), first identified in the ciliate Tetrahymena (Greider and Blackburn, *Cell 43:*405-413 (1985)). The telomerase enzyme is essential for complete replication of telomeric DNA because the cellular DNA-dependent DNA polymerases are unable to replicate the ultimate ends of the telomeres due to their requirement for a 5' RNA primer and their unidirectional mode of synthesis. Removal of the most terminal RNA primer following priming of DNA synthesis leaves a gap that cannot be replicated by these polymerases (Olovnikov, *Dokl. Akad. Nauk SSSR 201:*1496-1499 (1971); Watson, *Nature New Biol. 239:*197-201 (1972)). Telomerase surmounts this problem by *de novo* addition of single-stranded telomeric DNA to the ends of chromosomes (Greider and Blackburn, *Cell 43:*405-413 (1985),

Greider and Blackburn, *Nature 337*:331-337 (1989); Yu *et al.*, *Nature 344*:126-132 (1990); Greider, In *Telomeres*, eds. Blackburn and Greider, Cold Spring Harbor Laboratory Press, pp. 35-68 (1995)).

The telomerase enzymes that have been characterized to date are RNA-dependent DNA polymerases which synthesize the telomeric DNA repeats using an RNA template that exists as a subunit of the telomerase holoenzyme (Greider, In *Telomeres*, eds. Blackburn and Greider, Cold Spring Harbor Laboratory Press, pp. 35-68 (1995)). The genes specifying the RNA subunits of telomerases have been cloned from a wide variety of species, including humans (Feng *et al.*, *Science 269*:1236-1241 (1995); Greider, In *Telomeres*, eds. Blackburn and Greider, Cold Spring Harbor Laboratory Press, pp. 35-68 (1995)) and been shown in several instances to be essential for telomerase function *in vivo* (Yu *et al.*, *Nature 344*:126-132 (1990); Yu and Blackburn, *Cell 67*:823-832 (1991); Singer and Gottschling, *Science 266*:404-409 (1994); Cohn and Blackburn, *Science 269*:396-400 (1995); McEachern and Blackburn, *Nature 376*:403-409 (1995)). In addition, three proteins have been identified to date that are associated with telomerase activity. p80 and p95 were purified from the ciliate Tetrahymena (Collins *et al.*, *Cell 81*:677-686 (1995)) and the gene encoding a mammalian homologue of p80, TP1/TLP1, has also been cloned (Harrington *et al.*, *Science 275*:973-977 (1997); Nakayama *et al.*, *Cell 88*:875-884 (1997)). The specific mechanism by which these proteins participate in telomerase function has not been defined.

Perturbing the function of telomerase in the ciliate Tetrahymena, through the overexpression of an inactive form

of the telomerase RNA, or in yeast, through the mutation of genes encoding either the catalytic protein or template RNA subunit, leads to progressive telomere shortening as cells pass through successive cycles of replication (Yu *et al.*, *Nature 344*:126-132 (1990); Singer and Gottschling, *Science 266*:404-409 (1994); McEachern and Blackburn, *Nature 376*:403-409 (1995); Lendvay *et al.*, *Genetics 144*:1399-1412 (1996); Counter *et al.*, *Proc. Natl. Acad. Sci., USA 94*:9202-9207 (1997); Lingner *et al.*, *Science 276*:561-567 (1997)). This loss of telomeric DNA is ultimately lethal if it is not overcome. The lethality seems to be triggered when telomeres have been truncated below a critical threshold level. Hence, in the absence of compensating mechanisms, yeast cell lineages that lack telomerase activity have a lifespan dictated by the lengths of their telomeres.

In humans, telomerase activity is readily detectable in germline cells and in certain stem cell compartments. However, enzyme activity is not detectable in most somatic cell lineages (Harley *et al.*, *Cold Spring Harb. Symp. Quant. Biol. 59*:307-315 (1994); Kim *et al.*, *Science 266*:2011-2015 (1994); Broccoli *et al.*, *Proc. Natl. Acad. Sci., USA 92*:9082-9086 (1995); Counter *et al.*, *Blood 85*:2315-2320 (1995); Hiyama *et al.*, *J. Immunol. 155*:3711-3715 (1995)). Consistent with this, telomeres of most types of human somatic cells shorten with increasing organismic age and with repeated passaging in culture, similar to the situation seen in protozoan and yeast cells that have been deprived experimentally of a functional telomerase enzyme (Harley *et al.*, *Nature 345*:454-460 (1990); Hastie *et al.*, *Nature 346*:866-868 (1990)).

Eventually the proliferation of cultured human cells will halt at a point termed senescence (Hayflick and Moorhead, *Exp. Cell. Res. 25*:585-621 (1961); Goldstein, *Science 249*:1129-1133 (1990)), apparently before the telomeres of these cells have become critically short.

Cultured normal human cells can circumvent senescence and thereby continue to proliferate when transformed by a variety of agents. In such cultures, telomere shortening continues until a subsequent point is reached that is termed crisis, where telomeres have become extremely short (Counter *et al.*, *EMBO J. 11*:1921-1929 (1992) and *J. Virol. 68*:3410-3414 (1994); Shay *et al.*, *Oncogene 8*:1407-1413 (1993); Klingelhutz *et al.*, *Mol. Cell. Biol. 14*:961-969 (1994). Crisis, perhaps best described in SV-40 transformed cells, is characterized by karyotypic instability, particularly the types of instability observed in chromosomes lacking functional telomeres, and by significant levels of cell death (Sack, *In Vitro 17*:1-19 (1981)). The crisis phenotype is reminiscent of that observed in yeast and Tetrahymena cells in which telomerase function has been experimentally perturbed.

The simplest interpretation of these data is that the lifespan of telomerase-negative human cells, like that of their yeast and ciliate counterparts, is ultimately limited by the length of telomeres. Rare human cells that have acquired the ability to grow indefinitely emerge from crisis populations with a frequency of 10^{-6} to 10^{-7} (Huschtscha and Holliday, *J. Cell Sci. 63*:77-99 (1983); Shay and Wright, *Exp. Cell Res. 184*:109-118 (1989)). This implies that a mutational event is required to confer the immortal phenotype on these cells. The immortal cells that escape crisis are characterized by readily detectable

levels of telomerase activity and by stable telomeres (Counter *et al.*, *EMBO J. 11*:1921-1929 (1992) and *J. Virol. 68*:3410-3414 (1994); Shay *et al.*, *Mol. Cell. Biol. 15*:425-432 (1995); Whitaker *et al.*, *Oncogene 11*:971-976 (1995); Gollahon and Shay, *Oncogene 12*:715-725 (1996); Klingelhutz *et al.*, *Nature 380*:79-82 (1996)). This suggests that activation of telomerase can overcome the limitations imposed by telomere length on the lifespan of cell lineages.

Activation of telomerase also appears to be a major step in the progression of human cancers. Unlike normal human cells, cancer cells can be established as permanent cell lines and, thus, are presumed to have undergone immortalization during the process of tumorigenesis. Moreover, telomerase activity is readily detected in the great majority of human tumor samples analyzed to date (Counter *et al.*, *Proc. Natl. Acad. Sci., USA 91*:2900-2904 (1994); Kim *et al.*, *Science, 266*: 2011-2015 (1994); Shay and Bacchetti, *Eur. J. Cancer 33*:787-791 (1997)).

Taken together, these various observations have been incorporated into a model which proposes that the limitation on prolonged cell replication imposed by telomere shortening serves as an important anti-neoplastic mechanism used by the body to block the expansion of pre-cancerous cell clones. According to such a model, tumor cells transcend the crisis barrier and emerge as immortalized cell populations by activating previously unexpressed telomerase, enabling them to restore and maintain the integrity of their telomeres (Counter *et al.*, *EMBO J. 11*:1921-1929 (1992) and *Proc. Natl. Acad. Sci., USA 91*:2900-2904 (1994); Harley *et al.*, *Cold Spring Harb. Symp. Quant. Biol. 59*:307-315 (1994)).

A major question provoked by this model is the mechanism used to resurrect telomerase expression during tumor progression. Expression of the telomerase-associated protein TP1/TLP1 does not reflect the level of telomerase activity (Harrington *et al.*, *Science 275*:973-977 (1997); Nakayama *et al.*, *Cell 88*:875-884 (1997)). It is also clear that the levels of the human telomerase RNA component, *hTR*, cannot completely explain the regulation of telomerase activity. Although the levels of *hTR* and its mouse counterpart, *mTR*, increase with tumor progression (Feng *et al.*, *Science 269*:1236-1241 (1995); Blasco *et al.*, *Nat. Genet. 12*:200-204 (1996); Broccoli *et al.*, *Mol. Cell. Biol. 16*:3765-3772 (1996); Soder *et al.*, *Oncogene 14*:1013-1021 (1997)), the amounts of these transcripts do not always correlate with enzymatic activity. Indeed, *hTR* or *mTR* transcript levels can be significantly higher in telomerase-negative cells and tissues than in telomerase-positive cancer cells (Avilion *et al.*, *Cancer Res. 56*:645-650 (1996); Bestilny *et al.*, *Cancer Res. 56*:3796-3802 (1996); Blasco *et al.*, *Nat. Genet. 12*:200-204 (1996)). Similarly, even though telomerase levels increase 100- to 2000-fold during the immortalization of human cells, the level of *hTR* message increases at most two-fold (Avilion *et al.*, *Cancer Res. 56*:645-650 (1996). Therefore, derepression of the *hTR* and TP1 subunits cannot easily be invoked to explain the appearance of telomerase activity in the great majority of human tumor samples. Thus far, the rate-limiting step in telomerase activation has remained elusive.

Described herein is work which has shown that mutations in a gene of eukaryotic origin (in an open

reading frame required for telomere maintenance) results in loss (absence) of telomerase enzymatic activity in cells and that the encoded protein is physically associated with the active telomerase enzyme, thus showing that the DNA encodes a component of a eukaryotic telomerase holoenzyme. Also described herein is isolated DNA of eukaryotic origin which is required for telomerase enzymatic activity and the isolated encoded RNA transcripts and proteins. Specifically, *EST2*, a yeast gene, and *hEST2*, which is of human origin and the encoded respective transcripts and proteins (yeast EST2p and human hEST2) are described, as are their respective sequences. DNA of the present invention includes cDNA, genomic DNA, recombinantly produced DNA and chemically synthesized DNA. It can be obtained from a source in which it occurs in nature or can be produced using known chemical or recombinant DNA methods. The following is a discussion of work carried out relating to yeast and human telomerase protein.

Gene Encoding Protein Subunit Of Yeast Telomerase Holoenzyme and the Encoded Protein

As described herein, it has been shown, using a genetic screen in budding yeast, that mutations in *EST2*, an open reading frame required for telomere maintenance, (Lendvay, T.S. *et al.*, *Genetics 144*, 1399-1412 (1996) result in the absence of telomerase enzymatic activity in cells. As also described herein, epitope tagging and immunochemical studies showed that the Est2 protein is physically associated with the active telomerase enzyme. Thus, *EST2* encodes a component of the yeast telomerase holoenzyme.

Interest in telomeres and telomerase has heightened in recent years with the discovery that telomerase is present

at low, almost undetectable, levels in most human somatic tissues and is readily detectable in germline cells and in the vast majority of tumor cell samples (Counter, C.M. *et al.*, *Proc. Natl. Acad. Sci. USA 91*, 2900-4 (1994); Kim, N.W. *et al.*, *Science 266*, 2011-5 (1994). Somatic cell lineages which lack telomerase lose telomere segments progressively as they pass through successive replication cycles, limiting their lifespan (Harley, C.B. *et al.*, *Nature 345*, 458-60 (1990), Hastie, N.D. *et al.*, *Nature 346*, 866-8 (1990)). Conversely, cell populations that resurrect telomerase expression ensure maintenance of telomere length, thereby removing a barrier to their further unlimited replication and resulting in the process termed immortalization (Counter, C.M. *et al.*, *Embo. J. 11*, 1921-9 (1992)). These observations have led to a model which proposes that the process of telomere shortening limits the replicative potential of most human somatic cell lineages, and that cancer cells overcome this limitation by activating telomerase expression during the course of tumor progression (Counter, C.M. *et al.*, *Embo. J. 11*, 1921-9 (1992); Harley, C.B. *et al.*, *Cold Spring Harb. Symp. Quant. Biol. 59*, 307-15 (1994)).

Validation of this model has been held back by the difficulties associated with isolating and characterizing the telomerase enzyme and its encoding genes. Much of this work has focused on the telomerase enzymes of ciliates; the formation of thousands of mini-chromosomes present in their macro-nuclei seems to require correspondingly high amounts of telomerase, dwarfing the amounts available for study in metazoan cells. The telomerase enzyme in *Tetrahymena* has been reported to be composed of two proteinaceous and one RNA subunit, the latter responsible for templating the

telomeric DNA segments (Greider, C.W. & Blackburn, E.H., *Nature 377*, 331-7 (1989); Collins, K. *et al.*, *Cell 81*, 677-86 (1995); Yu, G.L. *et al.*, *Nature 344*, 126-32 (1990)). The protein subunits of telomerase have not been described in any other organism, although the genes specifying the RNA subunits of telomerase have been cloned in a wide range of other species (Blasco, M.A. *et al.*, *Science 269*, 1967-70 (1995); Feng, J., *et al.*, *Science 269*, 1236-41 (1995); Lingner, J. *et al.* , *Genes Dev. 8*, 1984-98 (1994); McEachern, M.J. & Blackburn, E.H., *Nature 376*, 403-9 (1995); Melek, M. *et al.*, *Mol. Cell Biol. 14*, 7827-38 (1994); Shippen-Lentz, D. & Blackburn, E.H., *Science 247*, 546-52 (1990); Singer, M.S. & Gottschling, D.E., *Science 266*, 404-9 (1994)).

Genetic strategies designed to identify and isolate telomerase-encoding genes have centered on the yeast, *S. cerevisiae*. Mutations in several genes have been found to lead to progressive telomere shortening. Of these genes, *TLC1*, which specifies the RNA subunit of the telomerase enzyme (Singer, M.S. & Gottschling, D.E., *Science 266*, 404-9 (1994)), is required for telomerase enzymatic activity (Cohn, M. & Blackburn, E.H., *Science 269*, 396-400 (1995); Lin, J.J. & Zakian, V.A., *Cell 81*, 1127-35 (1995); Lue, N.F. & Wang, J.C., *J. Biol. Chem. 270*, 21453-6 (1995); Steiner, B.R. *et al.*, *Proc. Natl. Acad. Sci. USA 93*, 3817-21 (1996)). On the other hand, *EST2* encodes a protein of unknown function (Lendvay, T.S. *et al.*, *Genetics 144*, 1399-1412 (1996)); *EST1* (Lundblad, V. & Szostak, J.W., *Cell 57*, 633-43 (1989)) and *CDC13* (Garvik, B. *et al.*, *Mol. Cell Biol. 15*, 6128-38 (1995); Nugent, C.I., Hughes, T.R., Lue,

N.F. & Lundblad, V., *Science 274*, 249-52 (1996)) are not essential for telomerase activity (Cohn, M. & Blackburn, E.H., *Science 269*, 396-400 (1995); Nugent, C.I. *et al.*, *Science 274*, 249-52 (1996)); and *KEM1* appears to encode a nuclease that acts on telomeres (Liu, Z. & Gilbert, W., *Cell* 77, 1083-92 (1994); Liu, Z. *et al.*, *Proc. Natl. Acad. Sci. USA 92*, 6002-6 (1995)). A search for genes encoding telomerase protein subunits was initiated because the protein subunits of the yeast telomerase have not yet been identified, and because there are no obvious homologs of the *Tetrahymena* telomerase protein subunits encoded in the yeast genome (Collins, K. *et al.*, *Cell 81*, 677-86 (1995); Cherry, J.M. *et al.*, Saccharomyces Genome Database (http// genome-www.stanford.edu/Saccharomyces).

A genetic screen designed to identify the genes specifying telomerase subunits was undertaken, based on the observation that telomere loss in yeast cells lacking the telomerase RNA component can be compensated by the actions of a second telomere-maintenance system that utilizes the DNA recombination machinery. An essential component of this machinery is the product of the *RAD52* gene. Accordingly, the simultaneous inactivation of *RAD52* and the telomerase RNA component results in cell lethality in both *S. cerevisiae* and *K. lactis* (Lendvay, T.S. *et al.*, *Genetics 144*, 1399-1412 (1996); McEachern, M.J. & Blackburn, E.H., *Genes Dev. 10*, 1822-34 (1996)). This lethality is only observed after some delay and occurs in concert with the extensive telomere shortening observed following serial passaging of the mutant cells. Applicants reasoned that mutation of other genes contributing to telomerase function, including the genes encoding the protein

subunit(s) of the enzyme, might be recognized because they similarly render the yeast dependent on wild-type *RAD52* for prolonged viability. If so, telomerase mutant yeast could be identified by their requirement for *RAD52* function.

A yeast strain carrying an inactive chromosomal *rad52Δ* allele that was complemented by a plasmid-borne wild-type *RAD52* gene linked to a *URA3* gene was generated, in order to determine if mutants in telomerase function could be revealed by a screen for mutants that require *RAD52* function. When *RAD52* function was inactivated through eviction of the *RAD52*-encoding plasmid (by counter-selecting against the URA3 marker with 5-fluoro-orotic acid (FOA), cell viability was unaffected for the succeeding 80 cell generations (Boeke, J.D. *et al.*, *Mol. Gen. Genet. 197*, 345-6 (1984). However, when the *TLC1* gene, which encodes the RNA subunit of telomerase, was additionally inactivated in this strain, a decrease in cell viability was observed after 60 generations. Moreover, when the rescuing *RAD52* plasmid was evicted, there was a marginal decrease in viability after 40 generations and complete inviability after 60 generations. This showed that telomerase-deficient cells perish upon the loss of *RAD52* function and that this phenotype is therefore useful to screen for mutants carrying lesions in a variety of genes affecting telomerase function.

The *rad52Δ* mutant cells carrying the complementing *RAD52* plasmid were mutagenized by homologous recombination with a library of yeast genomic fragments, each of which carried one or more copies of an inserted mini-Tn3::*lacZ*::*LEU2* transposon (Burns, N., *et al.*, *Genes Dev. 8*, 1087-105 (1994)). To ensure a high density of mutations,

$1x10^6$ transposon-mutagenized yeast clones were generated. These mutagenized yeast cells were cultured for 40 generations to allow the telomeres of any cells lacking telomerase function to shorten substantially. A portion of the cell colonies was replica-plated and the *RAD52* plasmid was evicted from one of each replica pair to identify cell clones that required *RAD52* for continued viability.

Of the 10^5 transposon-mutagenized clones plated after 40 generations, approximately 2,500 reproducibly lost viability following removal of the *RAD52* plasmid. In this case, as earlier, these cells were placed in the presence of FOA to select against the presence of the plasmid-borne *URA3* gene. The loss of viability following FOA might have derived through a second, unrelated mechanism in which the *URA3* gene became stabilized through chromosomal integration, rendering cells FOA-sensitive independent of any effects on telomeres. For this reason, the FOA-sensitive cell clones were mated with wild-type yeast, yielding diploids; all cell clones for which the diploid derivatives remained FOA-sensitive were discarded.

The 245 remaining *RAD52*-dependent cell clones were tested for changes in telomere structure by Southern hybridization analysis of their telomeres. Of these 245 haploids, 16 cell clones carried short telomeres identical to those seen in yeast lacking a functional *TLC1* gene. Tetrad analysis of the diploid derivatives of these clones was performed to determine whether the *rad52* synthetic lethal phenotype was caused by the transposon insertion or by an adventitious unlinked mutation elsewhere in the yeast genome. In each case, the lethality co-segregated with the transposon insertion, demonstrating that this phenotype is

derived directly from the genetic disruption effected by a single inserted transposon.

To determine which genes were disrupted by transposon insertion in the 16 yeast strains containing short telomeres, the transposons together with flanking genomic DNAs from each strain were cloned and the DNA sequences flanking the transposon insertion site were analyzed. This sequencing revealed that 6 clones represented 6 distinct insertion sites within the *TLC1* gene which, as mentioned above, specifies the RNA subunit of the telomerase. This result provided strong and direct validation of the utility of the strategy described to screen for genes that directly affect telomerase function.

Eight of the cell clones having shortened telomeres and lethality in the absence of *RAD52* carried transposons that had inserted into seven distinct positions within an uncharacterized open reading frame on chromosome XII at nucleotides 766540 to 769194 (Cherry, J.M. *et al.*, Saccharomyces Genome Database (http//genome-www.stanford.edu/Saccharomyces)).

The predicted amino acid sequence encoded by the gene identified on chromosome XII is presented in Figures 1A and 1B (SEQ ID NO.: 2); the predicted protein includes 884 amino acids with an estimated mass of 102 kilodaltons. The predicted gene product does not share significant sequence similarity with any sequences in the databases available through the National Center for Biotechnology Information BLAST server. However, subsequent to completion of the work described herein, a report was published on the identification of a novel gene, *EST2*, involved in telomere length regulation. The open reading frame identified on chromosome XII is identical to *EST2*; therefore, this gene is referred to herein as *EST2*.

Genetic analysis of the *est2* transposon-insertion mutants shows that they have the same delayed cell death phenotype, dependence on *RAD52*, and telomere shortening as was displayed by cells carrying mutations in their *TLC1* gene. Diploids heterozygous for disrupted alleles of *RAD52* and *EST2* were sporulated and the resulting tetrads were analyzed for growth in culture and for telomere length. In 33 tetrads analyzed, the vast majority of the *est2*::TN3 *rad52Δ* double mutant spore products became inviable by approximately 20 generations in culture, and the remainder died by approximately 40 generations. The viability of the *est2*::Tn3 mutants with wild-type *RAD52* began to decline by approximately 60 generations, and this phenotype was more pronounced after approximately 80 generations. The *EST2* wild-type spore products remained completely viable at all generations, regardless of their *RAD52* genotype. Consistent with the role of *EST2* in telomere maintenance, the decrease in telomere length, by which this gene was identified, co-segregated with the transposon insertional mutations in *EST2*. This telomere shortening was comparable to that observed in *tlc1*::TN3 mutants.

Until now, *TLC1* has been the only yeast gene known to be a subunit of telomerase and to be required for telomere activity *in vitro*. The *est2*::TN3 gene mutants are phenotypically indistinguishable from *tlc1*::TN3 mutants. Analyses were carried out to determine whether *EST2* is also required for telomerase function.

A telomerase assay was used to show that extracts from wild-type yeast catalyze the elongation of a single-stranded telomeric primer by four to sixteen nucleotides in an RNase-sensitive fashion, indicating telomerase activity.

However, extracts derived from yeast bearing transposons inserted into *EST2* lacked detectable telomerase activity, like *tlc1Δ* mutants, which are known to be telomerase-negative (Cohn, M. & Blackburn, E.H., *Science 269*, 396-400 (1995)). Comparison with dilutions of wild-type extract made it possible to estimate that extracts from both *tlc1* and *est2* mutants contain at most 5% of wild-type telomerase activity. While the actual levels of telomerase in the mutant yeast are likely to be much lower, such lower levels could not be quantified with this assay. As expected from the phenotypic analysis of tetrads, the absence of detectable telomerase activity co-segregated with transposon insertion, independent of the *RAD52* allele.

The requirement of *EST2* function for telomerase activity suggests that Est2 is either a protein subunit of telomerase or an upstream regulator required for telomerase activity. It was possible to distinguish between these possibilities by replacing the endogenous *EST2* gene with a variant encoding a protein with three influenza hemagglutinin (HA) epitope stages at its carboxy terminus (*EST2-HA*). This modified *EST2* allele and, thus, its product, is fully functional. The *EST2-HA* yeast show no overt phenotype, their telomeres are wild-type length, and extracts from these yeast exhibit normal levels of telomerase activity. Extracts from *EST2-HA* and control yeast were incubated with excess anti-HA antibody, after which the immunoprecipitates and resultant supernatants were separated and assayed for telomerase activity. The anti-HA antibody immunoprecipitated telomerase activity from extracts of yeast expressing the tagged Est2-HA protein, but not from extracts expressing normal Est2 protein. The immunoprecipitation was specific for the

anti-HA antibody since a control anti-Myc epitope antibody failed to immunoprecipitate activity from either extract. Essentially all of the telomerase activity remained in the supernatant of the anti-HA immunoprecipitation from wild-type extracts, whereas incubation with the anti-HA antibody almost completely immunodepleted the telomerase activity from the Est2-HA extracts. These data indicated that Est2 is not only physically associated with telomerase, but is a constituent of most, if not all, of the active telomerase complex.

Intriguingly, *EST2* shares no homology with either of the two genes reported to encode the *Tetrahymena* telomerase protein subunits (Collins, K. *et al.*, *Cell 81*, 677-86 (1995)). A search of the yeast genome (Cherry, J.M. *et al.*, Saccharomyces Genome Database (http//genome-www.stanford.edu/Saccharomyces) revealed no open reading frame sharing obvious sequence similarity with the *Tetrahymena* genes. Thus, the genes encoding the yeast and ciliate telomerase enzymes may be highly diverged, the Est2 function may be absent in *Tetrahymena*, or the analogous Est2 protein of *Tetrahymena* may not yet have been identified.

Work described herein supports the essential role of *EST2* gene for telomerase activity *in vitro* and *in vivo*. Immunochemical experiments show that Est2-containing complexes contain telomerase activity, and that removal of these complexes from the cell concomitantly removes the telomerase activity. These characteristics argue that Est2 is an essential and integral component of the yeast telomerase holoenzyme. The identification of this protein subunit of yeast telomerase should aid in the characterization of the other elements of this complex,

both in yeast and in other species, as well as in the understanding of the biochemical mechanism by which this enzyme acts.

Work described herein provides the basis for a method of assessing the ability of a molecule or compound to act as a telomerase inhibitor or as a recombination inhibitor. It provides a method by which a drug (molecule or compound) which inhibits telomerase or inhibits recombination can be identified. Eukaryotic linear chromosomes are capped with telomeres, a DNA-protein complex that protects the integrity of the chromosome end. Telomeric DNA sequences are composed of multiple repeat elements. When the telomeric DNA is lost from chromosome ends, cells die because of the resulting chromosomal instability.

Telomere length is maintained by two mechanisms. In almost all eukaryotes growing under normal conditions, telomeric DNA repeats are synthesized by the ribonucleoprotein enzyme telomerase. This RNA-dependent DNA polymerase (or "reverse transcriptase") copies the DNA repeats from an RNA template that is a component of the enzyme.

When the telomerase pathway is inactive, however, telomere length can be maintained by an alternative mechanism in yeast and possibly also in humans: the homologous recombination pathway used for double strand break repair.

In the yeast *Saccharomyces cerevisiae*, mutants of the telomerase subunits in conjunction with mutants of the recombination pathway have been shown to become inviable after growth for 40 to 80 generations. This synthetic lethal phenotype was used as a screen for telomerase mutants in a background of recombination mutants and, as a result, a novel telomerase gene was identified.

Drug screens which work in the same fashion as demonstrated herein for the genetic screen are useful to identify telomerase inhibitors in a recombination mutant background, and recombination inhibitors in a telomerase mutant background.

Telomere maintenance inhibitors, which can be a telomerase inhibitor alone, a recombination inhibitor alone, or a combination, can be identified by the method described herein, using appropriate mutant eukaryotic cells, such as the mutant yeast strain described herein. Such telomerase inhibitors can be further assessed, using known methods, to confirm their effectiveness as anti-fungal drugs or as anti-cancer drugs. Normal human somatic cells lack a pathway to maintain telomere lengths, and their telomeres shorten continuously. Immortal human cells, whether in tissue culture or the vast majority of malignant tumors in the human body, have developed a mechanism to maintain telomeres. Telomere maintenance occurs as a result of activation of the telomerase enzyme, activation of a recombination pathway, or possibly both. In yeast, the inactivation of both pathways has been shown to kill cells; in immortal tumor cells, pharmacological inhibitors must be tested before the effects of inactivation of either or both pathways can be assessed. A brief description of screens for telomerase inhibitors and recombination inhibitors follows.

Screen for Telomerase Inhibitors

As described below, a mutant strain of the yeast *Saccharomyces cerevisiae* that requires telomerase activity for viability has been generated. This strain can be used in a rapid and automatable biological assay for telomerase inhibitors.

In the mutant yeast strain, named DN, the *RAD52* recombination gene has been replaced by a *HIS3* gene. Disruption of the *TLC1* telomerase RNA subunit gene or the *MIT1/EST2* telomerase protein subunit gene in the *rad52* mutants has been shown to lead to a delayed cell death. Yeast lacking both recombination activity and telomerase activity die after about 20 to 60 cell generations.

Telomerase activity can be disrupted by enzyme inhibitors as well as by genetic inactivation, and, therefore, the mutant strains described can be used to identify telomerase inhibitors. For a telomerase inhibitor screen, test compounds are added at different concentrations to the DN mutant strain and to a wild-type control strain. Each day, the cultures are be diluted (e.g., 1:1000). Compounds that kill the yeast instantly, or that kill the DN and wild-type yeast with identical delays, are eliminated. Compounds that kill the DN yeast significantly before the wild-type yeast are telomerase inhibitors.

The method described is a biological assay and has several advantages over available methods. It is fast, automatable, and can be performed easily in large quantities. As a biological assay, it reflects the physiological function of the drugs. Furthermore, it allows testing of biochemical and uptake parameters in a single step. Finally, this assay will also exclude inhibitors of other metabolic enzymes, such as RNA and DNA polymerases and is highly specific for telomerase inhibitors.

High-throughput screening can be accomplished by growing the yeast in microtiter dishes. Aliquoting of media, dilution of test drug compounds, and dilution of yeast cultures can be performed robotically. Growth can be

measured by the optical density of the yeast culture at 595-600 nm; growth measurements and preliminary analysis of the data can also be automated.

The telomerase inhibitor screen has several applications, such as to identify inhibitors of fungal telomerase enzymes, mammalian telomerase enzymes and reverse transcriptase.

1) Inhibitors of fungal telomerase enzymes. This screen is a rapid method for identification of compounds that inhibit telomerase in the yeast *Saccharomyces cerevisiae*. This yeast is closely related to the fungal pathogen *Candida albicans* and, therefore telomerase inhibitors effective in *Saccharomyces* will likely function in *Candida*, as well as in other pathogenic yeast. There are only a few effective anti-fungal drugs currently available; many are inadequate because of their serious side effects. Thus, a novel approach to anti-fungal drugs could be clinically valuable for the treatment of systemic fungal infections.

2) Inhibitors of mammalian telomerase enzymes. Telomerase function is likely to be conserved between diverse species. Therefore, it is highly possible that inhibitors of yeast telomerase could also inhibit mammalian telomerase, and can be tested for their abiltiy to act as anti-cancer agents. These compounds can also serve as lead compounds for the identification of such inhibitors and for modification to produce more effective inhibitors (e.g., inhibitors which are longer-lived, exhibit great inhibitory effects, resist degradation by cellular enzymes).

3) Inhibitors of reverse transcriptase. The drugs that have been identified as telomerase inhibitors to date have been shown to be inhibitors of HIV reverse transcriptase. Telomerase inhibitors identified by the present screen can

be assessed, using known methods, for their ability to act as retroviral reverse transcriptase.

Screen for recombination inhibitors

In this case, inhibitors of the telomere recombination pathway are identified by their ability to kill telomerase mutant yeast but not wild-type yeast. The screen is similar to the telomerase inhibitor screen described above.

For a recombination inhibitor screen, test compounds are added at different concentrations to a telomerase mutant strain (e.g., *tlc1* or *mit1/est2*), referred to as a test strain, and to a wild-type control strain. Each day, the cultures are diluted 1:1000. Compounds that kill the yeast instantly, or that kill the telomerase mutant and wild-type yeast with identical delays, are eliminated. Compounds that kill the telomerase mutant yeast significantly before the wild-type yeast are recombination inhibitors. The ability of the compounds to inhibit recombination can be confirmed using known methods. The recombination inhibitors have several uses. For example, combination therapy of a recombination inhibitor with a telomerase for treating fungal infections and/or malignant neoplasms. In addition, recombination inhibitors can be used to potentiate the toxic effects of radiation treatment and of chemotherapeutic agents that induce DNA double-strand breaks.

The following methods and materials were used in the work described herein.

METHODS

Yeast strains: Strain DN was generated by replacing the *RAD52* coding sequence of the haploid strain L3853 (gift of G. Fink; genotype *MATa leu2-3,112 lys2-201 trp1-1 ura3-*

52 his3-200) with the *HIS3* gene using a PCR-based homologous gene disruption method (Baudin, A. *et al.*, *Nucleic Acids Res. 21*, 3329-30 (1993)). The absence of *RAD52* in this strain was complemented by introducing the plasmid pYPCR+[35], which encodes the *RAD52* gene under its own promoter and the selectable marker *URA3*, thereby generating strain DNR. The *TLC1* gene was replaced with the *LEU2* selectable marker by the above PCR procedure in L3853 and DNR cells, yielding the strains *tlc1Δ* and *DNRtlcΔ*, respectively.

Yeast mutagenesis: Approximately 230 μg of the appropriately prepared yeast::mini-Tn3::*lacZ*::*LEU2* genomic library (Burns, N., *et al.*, *Genes Dev. 8*, 1087-105 (1994)) were introduced by homologous recombination into approximately $7x10^6$ cells of strain DNR. Approximately $1x10^6$Ura$^+$Leu$^+$ clones representing successful transposon insertions were pooled and replated to a total number of $\sim 1x10^6$ clones to "age" the yeast for the *rad52Δ* synthetic lethal screen. $1x10^5$ of such yeast were then reseeded for the *rad52Δ* synthetic lethal screen, as described in the text. DNA flanking insertion sites were rescued as described by Burns, sequenced and compared to the *Saccharomyces* Genome Database (Stanford University School of Medicine, Department of Genetics) (Burns, N., *et al.*, *Genes Dev. 8*, 1087-105 (1994)).

Southern hybridization: Genomic DNA was isolated and digested with the restriction enzyme *Xho*I, which liberates the telomeric DNA (Guthrie, C., & Fink, G.R., *Guide to Yeast Genetics and Molecular Biology* (Academic Press, new York, 1991); Chan, C.S. & Tye, B.K., *Cell 33*, 563-73

(1983)). 2 μg of this DNA was resolved on 0.8% agarose gel for 570 Vh, and Southern hybridized as described by Counter *et al.* using a ^{32}P kinase labelled yeast telomeric oligonucleotide CACCACACCCACACACCACA (SEQ ID NO.: 37) (Counter, C.M., *et al.*, *Embo. J.* 11, 1921-9 (1992)).

Tetrad analysis: Diploid derivatives of the transposon-mutagenized yeast lacking the pYCPR+ plasmid were sporulated and tetrads were dissected according to standard methods (Guthrie, C., & Fink, G.R., *Guide to yeast genetics and molecular biology* (Academic Press, New York, 1991)). Presence of the *rad52::HIS3* allele and the miniTn3::*lacZ::LEU2* insertions were assayed on -His and -Leu synthetic complete plates, respectively.

Yeast extracts: 6 litres of yeast cultures were harvested at an optical density of 0.4-0.6 at 600 nm, resuspended in TMG buffer, disrupted with a bead beater and centrifuged for 90' at 100,000*g* at 4°C as described (Cohn, M. & Blackburn, E.H., *Science 269*, 396-400 (1995)). The supernatant was decanted, flash frozen and stored at -70°C. Extracts typically had a protein concentration of 16-20 mg/ml.

Telomerase assay: Telomerase activity was assayed essentially as outlined by Cohn and Blackburn with the following two modifications: first, 4 μg of crude S100 yeast extract was incubated with the oligonucleotide TGTCTGGGTGTCTGGG (SEQ ID NO.: 38) and second, the reaction products were purified and resolved on 15% acrylamide, 7M urea sequencing gels (Counter, C.M. *et al.*, *Proc. Natl. Acad. Sci. USA 91*, 2900-4 (1994)).

Epitope tagging: An *HA-URA3-HA* cassette was introduced into the 3' end of the *EST2* gene by homologous recombination, and then counter-selected on FOA to excise

the *URA3* gene and generate three copies of the influenza hemagglutinin tag in frame with *EST2* (Schneider, B.L. *et al.*, *Yeast 11*, 1265-74 (1995)). The construction of this epitope-tagged version deleted the 10 carboxy-terminal amino acids of Est2; these codons contain a TA repeat sequence that is present in multiple copies in the yeast genome.

Immunoprecipitation and immunodepletion: 540 μg of yeast extract was diluted in 170 μl of TMG buffer (Cohn, M. & Blackburn, E.H., *Science 269*, 396-400 (1995)) supplemented with 150 mM NaCl (buffer TMGN) and incubated at 4°C with 20 μl of packed protein A-agarose beads pre-loaded with anti-HA antibody (monoclonal antibody 12CA5). After 2 hours, the mixture was briefly centrifuged to separate the agarose beads from the supernatant extract. The beads were then washed four times with buffer TMGN and resuspended in 50 μl TMGN. As a control, a duplicate extract was diluted and left untreated on ice for 2 hours. 4 μg of supernatant or untreated extracts and 10 μl of beads were assayed for telomerase activity.

Human Telomerase Catalytic Subunit Gene and Encoded Protein

The findings presented herein support modulation of *hEST2 (hTERT)* RNA expression levels as an important mechanism used in a variety of developmental contexts to determine the amount of telomerase activity present in specific cell lineages. Indeed, the levels of the catalytic subunit of telomerase may represent a rate-limiting determinant of enzyme activity in many types of cells. Moreover, up-regulation of *hEST2* message may be an important mechanism through which telomerase becomes activated during both cellular immortalization and the progression of malignant tumors.

hEST2, (also referred to as hTERT) the human telomerase subunit described here, shares extensive sequence similarities with the catalytic subunits of the yeast and ciliate telomerase enzymes. The amino acid sequence conservation between these three enzymes is extensive and is scattered throughout their reading frames. In addition, no related genes were detected in the human genome by Southern blotting analysis. Together, these data support the conclusion that the gene described here encodes the catalytic subunit of the human telomerase holoenzyme. Further proof that this is the case can be obtained using known methods, such as experiments analogous to those performed with the yeast *EST2* gene, described herein and by Lingner and co-workers. (Lingner *et al.*, *Science 276*:561-567 (1997)). For example, experiments that will provide additional proof that the gene described herein encodes the human telomerase subunit include those that demonstrate that mice lacking the mouse *mEST2* homologue also lack telomerase activity; that the hEST2 protein is physically associated with a ribonucleoprotein complex that exhibits telomerase activity; and that alteration of critical residues in the domain of hEST2 that is homologous to RTs inactivates its catalytic function.

As described herein, three telomerases described to date (hEst2, yeast Est2 and Euplotes p123) include motifs that indicate they are distant homologues of a variety of RTs. They also share several unique, telomerase-specific sequence motifs. Yeast, ciliates and mammals represent highly diverged branches of the phylogenetic tree, which suggests that the catalytic subunit of the telomerase was developed early in eukaryotic evolution. It is possible that it was present in the cell that became ancestral to all contemporary eukaryotes. Even earlier, it appears that

this enzyme shares ancestry with the precursors of the RTs specified by a variety of transposons and viruses.

The repression of *hEST2* mRNA in telomerase-negative cells and tissues and its upregulation that is associated with a number of human tumor cell lines and primary human tumors suggest one mechanism by which telomerase activity might be modulated. During development, the expression of the *hEST2* mRNA may be repressed in many post-embryonic cell lineages, depriving cells in these lineages of the telomerase catalytic subunit. This in turn may underlie the observed progressive telomeric shortening associated with aging in the cells in many of these lineages.

In contrast, the reappearance of telomerase enzyme activity when transformed cells escape from crisis or when tumors progress toward malignancy may, in many cases, be explained mechanistically by the de-repression of *hEST2* mRNA expression. As shown here, in cell cultures, this de-repression occurs in both transformed embryonic kidney cells and lymphocytes when they emerge from crisis and begin to exhibit telomerase activity. Not addressed here is whether the enhanced *hEST2* RNA expression is achieved at the transcriptional or post-transcriptional level.

Described here is a correlation between *hEST2* mRNA levels and assayable telomerase activity. These two manifestations of *hEST2* gene expression are present in a constant, predictable ratio. This provides the basis for assessing cells for *hEST2* mRNA as an indicator of telomerase activity or assessing cells for telomerase activity as an indicator of *hEST2* mRNA occurrence or level. It is possible that other mechanisms besides the presently observed modulation of *hEST2* mRNA levels may intervene to modulate telomerase activity.

As described in Example 8, over expression of *hEST2* in previously (normally) telomerase-negative cells is sufficient to impart telomerase activity to these cells, providing clear proof that hEST2 is the human telomerase catalytic subunit. The data demonstrate that the ectopic expression of hTERT in otherwise telomerase-negative human cells is necessary and sufficient for induction of telomerase activity. In addition, the physical association of hTERT with telomerase activity confirms that hTERT is a telomerase subunit. Up-regulation of the *hTERT* gene is the sole barrier to activation of telomerase in the tested cells.

The identification of hEST2 as the gene specifying the telomerase catalytic subunit provides an entree into understanding one of the essential steps in human tumor pathogenesis -- that leading to cell immortalization. Enzymatic assays have demonstrated that telomerase is often activated at a relatively late step in tumor progression (Harley *et al.*, *Nature 345:*458-460 (1994)). This step may occur when evolving, pre-malignant cell clones have surmounted the senescence barrier and subsequently, having exhausted their telomeric ends, encounter the successive barrier of crisis. At this stage, the activation of telomerase may enable cells to breach these barriers to further clonal expansion, thereby conferring great selective advantage on the rare cell that has acquired the ability to resurrect the long-repressed telomerase activity. Indeed, activation of telomerase may represent an essential step in tumor progression.

Such dependence on telomerase activity means that this enzyme represents an attractive target of drugs designed to interfere with malignant cell proliferation. The finding that *hEST2* message is up-regulated in human tumors and in

immortalized cells lends further credence to this idea. Furthermore, the identification of hEST2 as the candidate telomerase catalytic subunit provides a biochemical reagent for identifying such drugs.

Described herein is a human gene, *hEST2*, which is a telomerase catalytic subunit gene. The gene transcript (mRNA) is expressed in primary human tumors, cancer cell lines and telomerase-positive tissues, but is undetectable, using the assays described herein, in cell lines known to be telomerase negative and in differentiated telomerase-negative tissues. The *hEST2* gene shares significant sequence similarity with a yeast telomerase catalytic subunit gene (S. cerevisiae EST2) and a ciliate telomerase catalytic subunit gene (Euplotes p123), as shown in Figure 2. The three telomerase enzymes are members of the reverse transcriptase family of enzymes. There are seven conserved sequence motifs which define the polymerase domains of reverse transcriptases and six of these domains are present in hEST2, including the invariant aspartic acid residues which are required for telomerase activity. Also the subject of the present invention is DNA which encodes hEST2 protein of SEQ ID NO.: 3; DNA which is the complement of DNA of SEQ ID NO.: 35; DNA which hybridizes to the complement of DNA of SEQ ID NO.: 35; DNA which hybridizes to the complement of DNA of SEQ ID NO.: 35 and encodes hEST2 protein of SEQ ID NO.: 3, DNA which is a gene which localizes to human chromosome 5, subband 5p15.33. A RNA transcript or RNA message encoded by hEST2 DNA of SEQ ID NO.: 35 or by DNA which encodes hEST2 protein of SEQ ID NO.: 3 is also the subject of this invention.

As shown in Figure 2, conceptual translation of the 4030 bp *hEST2* DNA shows an open reading frame of 1132 amino acids which is predicted to be an approximately 127 kDa

protein. As also shown in Figure 2, there is a clear relatedness among hEST2, yeast EST2 and Euplotes p123 in regions of the gene outside the RT motifs, particularly sequence identities in a region just before motif 1 of the hEST2 protein. This region of similarity appears unique to telomerases and, thus, can be used to produce reagents, such as antibodies reactive with the unique region and nucleic acid probes and primers useful to identify *hEST2* DNA, using, for example, hybridization or amplification methods. *hEST2* is a single-copy gene which is approximately 40 kb in size and was shown to localize (to be present on) chromosome 5, particularly subband 5p15.33. (See Figure 4) Expression levels in normal human tissues and human cancer cell lines were assessed, as described in Example 3. This assessment revealed two major RNA species (4.4 kb and 9.5 kb) and one minor RNA species (approximately 6 kb). *hEST* message was detectable in several normal tissues, including thymus, testis and intestine; the latter two tissues are known to be telomerase-positive and the telomerase status of thymus has not been reported. Using the assays described herein, *hEST2* transcript was not detected in most other normal human tissues, including heart, brain, placenta, liver, skeletal muscle and prostate, all of which have been reported to be telomerase-negative.

As described herein, the *hEST2* transcript or message (mRNA) is expressed in primary human tumors and cancer cell lines, but is not detectable in telomerase-negative cell lines and differentiated (normal, nontumorigenic) telomerase-negative tissues. Further, *hEST2* message is not detectable in pre-crisis, telomerase-negative transformed cells, but is readily detectable in post-crisis, telomerase-positive immortalized cells. The work described

herein provides a method of differentiating between telomerase-positive cells and telomerase-negative cells, as well as for monitoring a change in telomerase content of cells. As a result, as described below, the present work provides a method of identifying cells which are telomerase positive and, thus, of identifying cells which are tumor or cancer cells or are en route to becoming tumor cells (malignant).

A method of identifying telomerase-positive cells is the subject of the present invention. Also the subject of the present invention is a method of identifying cells which are transformed, malignant, cancerous, tumor or post-crisis cells or are en route to or likely to become transformed, malignant, cancerous, tumor or post-crisis cells. For convenience, the term malignant or malignancy is used herein to refer to transformed, cancer, cancerous, tumor, tumorigenic and post-crisis cells. For example, the phrase "a method of identifying malignant cells in an individual" encompasses a method of identifying cancer cells, transformed cells, tumor cells and/or post-crisis cells in an individual. The present invention, thus, provides a method of diagnosing, aiding in the diagnosis of or predicting an increased likelihood of the occurrence of cancer, tumor formation and/or the development of malignancy in an individual, particularly a mammal and, specifically, a human. In the method, telomerase content of cells is assessed by detecting or measuring *hEST2* DNA, *hEST2* transcript (*hEST2* message) or *hEST2*-encoded protein (hEST2 protein). The quantity of *hEST2* DNA, *hEST2* transcript or hEST2 protein can be determined; alternatively, the occurrence (presence or absence) of *hEST2* DNA, *hEST2* transcript or hEST2 protein can be detected. The presence of *hEST2* DNA, *hEST2* message and/or

hEST2 protein in cells is indicative of the presence of malignant cells or malignancy in the individual.

In one embodiment, *hEST2* DNA, *hEST2* RNA (or both) is detected or measured in tissue or cells obtained from an individual. This can be carried out using known methods, such as hybridization (e.g., *in situ* hybridization) or amplification methods. All or a portion of *hEST2* DNA or RNA can be used in such a method as a probe (e.g., to detect *hEST2* DNA or RNA) or a primer (e.g., to amplify *hEST2* DNA).

As described herein, up-regulation of *hEST2* RNA is associated with activation of telomerase during cell immortalization and may be an important mechanism through which telomerase becomes activated during cellular immortalization, the progression of malignant tumors or both. Thus, detection and/or measurement of *hEST2* RNA provides a means of assessing cells (e.g., mammalian, such as human cells) for malignancy or the likelihood of progression to malignancy. For example, detection of *hEST2* RNA, even without quantification of *hEST2* RNA levels, in cells indicates that telomerase is activated and that the cells are malignant or have an increased likelihood of progression to malignancy (relative to cells in which *hEST2* RNA is not detected). Quantification of *hEST2* RNA can be carried out as well. Higher concentrations of *hEST2* RNA cells are expected to indicate a more advanced stage in malignancy or a greater likelihood of progression to malignancy than is the case with lower *hEST2* RNA levels in cells. *hEST2* RNA levels determined by assessing an individual's cells can be compared with pre-established *hEST2* RNA levels. For example, *hEST2* RNA levels determined

for an individual's cells can be compared with *hEST2* RNA levels in cells known to be malignant and/or at established stages of malignancy (e.g., a pre-established reference or standard).

Alternatively, the levels of *hEST2* RNA determined for an individual can be compared with *hEST2* RNA levels determined for the same individual in prior assessments, in which case the individual serves as his or her own standard or reference. In this approach, it is possible to monitor (assess over time) the status of the individual. This is useful, for example, to assess or aid in assessing whether the malignancy or cancer is progressing, remaining unchanged or regressing in that individual. This is particularly useful in assessing the status of an individual's malignancy or cancer before, during and after therapy (e.g., chemotherapy, radiation, surgery) and to assess the therapeutic value or suitability of a drug(s) or other agent(s) for a specific individual in need of treatment. As mentioned, assessment of hEST2 RNA (or DNA) can be carried out using known methods, such as *in situ* hybridization, Rnase protection assay, reverse transcriptase (RT) PCR, Southern blot analysis or Northern blot analysis.

In one embodiment, the presence of malignancy or an increased likelihood of malignancy is assessed in a method in which a nucleic acid (e.g., DNA) anneals to nucleic acid (e.g., RNA). For example, the method can be Northern blot analysis, Rnase protection assay or RT PCR. One embodiment is as follows: Cells to be assessed for hEST2 DNA or RNA are obtained from the individual and processed to render DNA and/or RNA in the cells available for annealing with or hybridization of complementary nucleic acid sequences DNA or RNA (e.g., with complementary poly- or oligonucleotides

(DNA or RNA)). All or a portion of hEST2 DNA or hEST2 RNA can be used as the hybridization probe or sequence. As described herein, hEST2 protein shares sequence similarities and identities with yeast p123 and Euplotes Est2p, particularly in a region just before motif 1 (Figure 2, boxed region). As described, this region appears to be unique to telomerases. Thus, it is useful in assays which identify and/or quantify telomerase in cells and provide an assessment of malignancy or the likelihood of progression to malignancy of cells (e.g., from an individual in whom a diagnosis of malignancy or assessment of the likelihood of the occurrence of malignancy is needed). DNA or RNA comprising DNA or RNA encoding region(s) of similarity and/or identify with yeast p123 and/or Euplotes Est2p such as region(s) unique to telomerase, can be used in the hybridization assay or other assay carried out to detect or measure *hEST2* DNA or RNA in an individual's cells. Alternatively, DNA or RNA comprising DNA or RNA which encodes a region or regions unique to hEST2 protein can be used as the probe. In an alternative approach, the *hEST2* DNA or RNA is used as a primer in an amplification method. The cells processed to render DNA and/or RNA available for annealing (hybridization) with complementary poly- or oligonucleotides (processed cells) are combined with all or a portion of *hEST2* DNA or RNA under conditions appropriate for hybridization of complementary nucleic acids (DNA, RNA) to occur. Whether annealing (hybridization) occurs is then determined. If hybridization occurs (forming complexes of cellular DNA or RNA and the hEST2 poly- or oligonucleotides), it is an indication that the cells contain *hEST2* DNA or *hEST2* RNA. Poly- or oligonucleotides used in the method can be, for example, DNA which encodes the telomerase motif described herein (e.g., DNA which

encodes amino acid residues 556 to 565 of SEQ ID NO.: 3 or amino acid residues 560 to 565 of SEQ ID NO.: 3) or amino acid residues 1 to 50 of hEST2 protein or a portion thereof. If *hEST2* RNA is upregulated (expressed), it is indicative of activation of telomerase and of malignancy or an increased likelihood of progression to malignancy. The amount of *hEST2* RNA can also be determined (by determining, for example, the extent to which hybridization with *hEST2* DNA or RNA occurs) and used to assess the extent of telomerase activity and the stage of malignancy. This detection and/or measurement of *hEST2* DNA or RNA can be carried out at various intervals over time to assess the status of the malignancy (e.g., progression, reversal).

In another embodiment of assessing telomerase content and, thus, malignancy or cancer of cells, hEST2 protein is analyzed (detected and/or quantified). This can be done using known methods, such as enzymatic assays or immunoassays, which indicate hEST2 protein is present in cells assessed and, optionally, quantify the protein. For example, hEST2 telomerase activity can be assessed by determining whether extension of a telomeric primer occurs when a sample (e.g., cells, cell fractions or cell component(s)) from an individual is combined with the telomeric primer under conditions appropriate for hEST2 protein telomerase activity and telomeric extension. Alternatively, antibodies which recognize (bind) hEST2 protein can be used to determine if the protein is present in cells or other sample obtained from an individual. In the method, cells are obtained from an individual and processed or treated to render proteins in the cells available for binding with antibodies, thus producing processed cells. The processed cells are combined or contacted with antibodies which bind hEST2 protein and

whether binding occurs between antibodies which bind hEST2 protein (hEST2 protein-binding antibodies) and protein in the cells is determined. If hEST2 protein-binding antibody/protein binding occurs (to form complexes), it is indicative of the presence of hEST2 protein in the cells and, thus, of malignancy or an increased likelihood of progression to malignancy in the individual (in cells of the individual). The antibodies can be monoclonal antibodies or polyclonal antibodies (e.g., polyclonal sera) and can be specific for (bind or recognize only) hEST2 protein or can be nonspecific for hEST2 protein (bind or recognize hEST2 protein and other protein(s)). Humanized antibodies can also be used. Antibodies which recognize a motif or epitope unique to hEST2 and/or to members of the class to which hEST2 belongs are particularly useful. For example, antibodies which specifically bind the telomerase motif, represented in Figure 2, which is common to hEST2 protein, yeast p123 protein and/or Euplotes EST2 can be produced, using known methods, and used to assess cells for hEST2 protein. Alternatively, antibodies which specifically bind a motif which is unique to hEST2 protein (e.g., all or a portion of amino acid residues 1 to 50 of hEST2 protein) can be used. As discussed above with reference to *hEST2* DNA and *hEST2* RNA, the assessment can be detection (determination of presence or absence) or measurement (quantification). The presence of hEST2 protein is indicative of malignancy or at least of an increased likelihood of progression to malignancy.

Antibodies which bind or recognize (specifically or non-specifically) hEST2 protein are also a subject of the present invention. Nucleic acid probes and primers (poly- or oligonucleotides) comprising *hEST2* DNA or RNA are also the subject of this invention. The poly- or oligonucleotides will vary in length and need to be of

sufficient length to bind to and remain bound to *hEST2* DNA or *hEST2* RNA under the conditions used. They will generally be at least four to six bases in length and can comprise the entire *hEST2* DNA or *hEST2* RNA (alone or with additional non *hEST2* DNA or RNA). Preferably the probes or primers will hybridize to at least a characteristic portion of *hEST2* DNA or *hEST2* RNA (a portion which is present in members of the class to which hEST2 belongs), thus making it possible to identify *hEST2* DNA or *hEST2* RNA substantially to the exclusion of other proteins. For example, a probe or primer can comprise DNA or RNA which encodes a characteristic motif or region of hEST2 protein, such as the telomerase motif (e.g., amino acids 556 to 565 or 560 to 565) or the amino terminal amino acid sequence of hEST2 protein not present in Est2p or p123. (See Figure 2). For example, a probe or primer encoding amino acid residues 1 to 50 (inclusive) of hEST2 protein (SEQ ID NO.: 3) or a portion thereof can be used.

Methods of altering *hEST2* DNA transcription and expression, methods of altering hEST2 protein function and methods of identifying agents which alter (enhance or reduce) transcription, expression or function are also the subject of this invention. Also the subject of the present invention are a method of increasing or shortening the lifespan of cells in culture, *ex vivo* or *in vivo*; agents or drugs(DNA, RNA, drugs, small organic molecules, enzymes, for example) useful for lengthening or shortening cell lifespan and methods of identifying agents which enhance lifespan. For example, one embodiment of a method of inhibiting hEST2 protein comprises introducing into cells an agent which inhibits hEST2 protein, directly or indirectly; as a result, function of the enzyme is

abolished or the enzyme is inactivated. hEST2 protein is inhibited directly, for example, by introducing into cells an agent which binds to or otherwise "ties up" hEST2 protein such that it is less active or inactive (cellular telomerase activity is reduced or eliminated). Alternatively, a drug or agent which inactivates hEST2 or inhibits hEST2 catalytic function (and concomitantly reduces or eliminates telomerase activity) by degrading it or preventing it from being produced can be introduced into cells in which hEST2 protein is to be inhibited. Such drugs or agents can be, for example, a small organic molecule or a dominant negative protein. Agents which prevent production of *hEST2* message or its further processing (e.g., to produce DNA) or a dominant negative form of the telomerase protein can also be introduced into cells to inhibit hEST2 protein. Methods and agents which inhibit hEST2 catalytic function are useful for treatment of cancer and, thus, are useful as anti-cancer therapies.

If *hEST2* DNA, *hEST2* RNA or hEST2 protein is inhibited (partially or totally) in cells, lifespan of the cells will be shortened (shorter than it would be if the DNA, RNA or protein were not inhibited). For example, if a cell is malignant or more likely to progress to malignancy because of hEST2 function, an agent or drug which inhibits *hEST2* transcription or expression or hEST2 protein function in the cell will also shorten the lifespan of the cell because telomerase activity (and, thus, chromosomal extension or maintenance) will be inhibited. The agent is useful as a therapeutic for treating or preventing malignancy in individuals in whom malignant cells or cells with increased likelihood to progress to malignancy are present. These agents include, for example, enzyme (telomerase or hEST2 protein) inhibitors, enzymes which degrade hEST2 protein,

hEST2 transcriptional regulators, antisense molecules (e.g., DNA, RNA, PNA) and dominant negative mutant forms of hEST2. In a method of the present invention in which lifespan of cells is altered (increased or decreased) in an individual, a drug or agent is introduced into the individual in such a manner that it enters cells of the individual in sufficient quantity to have the desired effect (increase or decrease in cell lifespan). For example, a drug which prevents production of *hEST2* transcript of hEST2 protein function (directly or indirectly) is introduced into an individual, using known methods, in sufficient quantities to enter cells, such as a tumor, precancerous or cancerous cells, whose lifespan is to be decreased (e.g., made shorter than would be the case in the absence of the drug). Alternatively, a drug which enhances cell lifespan can be introduced into an individual in such a manner that it enters cells in sufficient quantity to enhance hEST2 protein expression or prolong its activity (e.g., by blocking its degradation).

The present invention is also a method of enhancing or increasing the lifespan of cells in culture, in which telomerase activity or function is enhanced, such as by introducing *hEST2* DNA or hEST2 protein into the cells or by activating an endogenous *hEST2* gene. For example, the lifespan of normal human or other mammalian cells can be extended (immortalized human or other mammalian cells can be produced) and the resulting cells used for therapeutic purposes (e.g., grafting of tissue (such as skin) or of organs), screening or assay methods or production of proteins or other cellular products. For example, the lifespan of epithelial cells, keratinocytes or endothelial cells can be extended by introducing *hEST2* DNA into the cells, in which the *hEST2* DNA is expressed or introducing

hEST2 protein into the cells. The resulting cells with longer lifespan can be transplanted into or grafted onto an individual (e.g., as skin grafts, as systems for delivery of therapeutic proteins, such as hormones and enzymes), to whom they provide therapeutic benefit. In the method of extending the lifespan of cultured cells, cells whose lifespan is to be extended are cultured under conditions appropriate for their viability and *hEST2* DNA or hEST2 protein is introduced into them. The resulting cells are maintained under conditions appropriate for expression of *hEST2* DNA and/or activity of hEST2 protein, with the result that the lifespan of the cells is enhanced. At an appropriate time (e.g., after sufficient cells with enhanced lifespan are available) enhanced lifespan cells (such as keratinocytes) are transplanted into an individual (e.g., as a skin graft). cells.

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLE 1: Cloning of *hEST2 (hTERT)*

The expressed sequence tag database (dbEST) was searched for sequences related to the yeast protein Est2p (Lendvay *et al.*, *Genetics 144*:1399-1412 (1996)) and the Euplotes protein p123 (Lingner *et al.*, *Science 276*:561-567 (1997)), using the program TBLASTN and the server http://www.ncbi.nlm.nih.gov/BLAST/. This resulted in the identification of a homologous expressed sequence tag, Genbank accession # AA281296, which derived from Soares NbHTGBC cDNA clone 712562. DNA from clone plasmid 712562 was PCR-amplified with primers HT-1 (5' -AAGTTCCTGCACTGGCTGATGAG- 3')(SEQ ID NO.: 39) and HT-5 (5' -TCGTAGTTGAGCACGCTGAACAG- 3')(SEQ ID NO.: 40). The

resulting 377 bp fragment was used to probe 1 ZAP phage cDNA libraries derived from the human Jurkat T-cell lymphoma (Stratagene, La Jolla, CA) and human Nalm-6 pre-B cell leukemia cell lines (Weissbach *et al.*, *J. Biol. Chem.* *269*:20517-20521 (1994)). A total of seven independent clones were isolated from these two libraries. Three of these cDNA clones together with plasmid 712562 were sequenced completely in both directions. The remaining clones were sequenced in specific regions. Reprobing of the Jurkat library with the 5'-most 500 bp region identified from the *hEST2* cDNA clones yielded one new clone containing an insert that overlapped with already determined sequences.

Rapid amplification of cDNA ends (RACE) was performed by PCR amplifying testis Marathon-ready cDNA (Clontech, Palo Alto, CA) with flanking primer AP-1 (Clontech) and *hEST2* primer R0096 (5' -CAAGAAACCCACGGTCACTCGGTCCACGCG-3')(SEQ ID NO.: 41), and then re-amplifying with flanking primer AP-2 and *hEST2* primer R0098 (5' -CAGCTCCTTCAGGCAGGACACCTGCGGG- 3')(SEQ ID NO.: 42). The product was subcloned and sequenced in both directions. RACE was also performed on aliquoted pools of plasmid DNA from a human testis cDNA library (Reduced Complexity cDNA Analysis, Qingyun Liu and Fang Chen, unpublished results) with primers HT-21 (5' -CAGGTGACACCACACAGAAA- 3') (SEQ ID NO.: 43) or HT-22 (5' -TTCCAAGCAGCTCCAGAAA- 3')(SEQ ID NO.: 44) and a vector primer, re-amplified with the vector primer and *hEST2* primers R0098 or R0097 (5' -CCTTCGGGGTCCACTAGCGTGTGGCGG)(SEQ ID NO.: 45), then purified and sequenced.

DNA sequencing reactions were performed with the AmpliTaq FS Prism ready reaction cycle sequencing kit (Perkin-Elmer/ABI) and electrophoresed on a 373 A Stretch

ABI DNA sequencer. The resulting sequences were assembled into a contig using the program Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI). The 1132 amino acid ORF identified in this contig was aligned with p123 and Est2p using the Pattern-Induced Multi-sequence Alignment program version 1.4 (R. F. Smith, Baylor College of Medicine & T. F. Smith, Boston University) using the server http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html, with minor modifications to the final alignment. BLASTP searches were performed against the non-redundant protein databases using the server http://www.ncbi.nlm.nih.gov/BLAST/.

EXAMPLE 2: Identification of the *hEST2* Gene

The sequences derived from the catalytic subunit of the S. cerevisiae telomerase, Est2p, and from the Euplotes p123 protein (Lendvay *et al.*, *Genetics 144*:1399-1412 (1996); Counter *et al.*, *Proc. Natl. Acad. Sci., USA 94*:9202-9207 (1997); Lingner *et al.*, *Science 276*:561-567 (1997)), were used to identify a human homologue (Genbank accession # AA281296) in the National Center for Biotechnology Information expressed sequence tag database. As shown in Figure 2 (underlined sequence), the conceptual translation of this DNA sequence shows clear relatedness to both the yeast and ciliate telomerase sequences as evidenced by identical or similar amino acid residues scattered throughout the entire length of the expressed sequence tag.

The identified expressed sequence tag provided only a fragment of the putative human telomerase open reading frame. Therefore, a human Jurkat T-cell lymphoma and a human Nalm-6 pre-B cell leukemia cDNA library were screened

with a probe derived from the expressed sequence tag. Seven cDNA clones were retrieved. The resulting sequence was extended further in the 5' direction by rapid amplification of cDNA ends (RACE) on human testis cDNA and on an independently generated human testis cDNA library.

Assembly of the cDNA clones and RACE products, together with the clone containing the expressed sequence tag, resulted in a contiguous sequence spanning 4030 bp. Conceptual translation of this 4 kbp sequence reveals an open reading frame of 1132 amino acids which is predicted to encode a 127 kDa protein (Figure 2). Although we have not identified an in-frame upstream stop codon in the sequence, the first methionine identified by RACE (Figure 2) is a candidate for the translation start site, as the sequence of this putative translation initiation site, CCCGCGAUGC (SEQ ID NO.: 46), is similar to the consensus GCC(A/G)CCAUGG (SEQ ID NO.: 47) characteristic of translation initiation sites (Kozak, *Cell* 44:238-292 (1986)).

The predicted 127 kDa protein shares extensive sequence similarity with the entire sequences of the Euplotes and yeast telomerase subunits (Figure 2) and extends beyond the amino- and carboxyl-termini of these proteins. A BLAST search reveals that the probabilities of these similarities occurring by chance are 1.3×10^{-18} and 3×10^{-13}, respectively. By way of comparison, the probability of similarity between the yeast and Euplotes telomerases in a protein BLAST search is 6.9×10^{-6}. The human gene was initially named *hEST2* (*human EST2* homologue) to reflect its clear relationship with the yeast gene, the first of these genes to be described. The current name for the gene is *hTERT*, which stands for human TElomerase Reverse Transcriptase. *EST2* was named because of the

phenotype of ever shortening telomeres caused by its mutant alleles (Lendvay *et al.*, *Genetics 144:*1399-1412 (1996)) and was later demonstrated to encode the yeast telomerase catalytic subunit (Counter *et al.*, *Proc. Natl. Acad. Sci., USA 94:*9202-9207 (1997); Lingner *et al.*, *Science 276:*561-567 (1997)).

Like the yeast and ciliate telomerase proteins, hEST2 (*hTERT*) is a member of the reverse transcriptase (RT) family of enzymes (Figures 2 and 3A-3F). Seven conserved sequence motifs, which define the polymerase domains of these enzymes, are shared among the otherwise highly divergent RT family (Poch *et al.*, *EMBO J. 8:*3867-3874 (1989); Xiong and Eickbush, *EMBO J. 9:*3353-3362 (1990)). p123 and Est2p share six of these motifs with, most prominently, the a2-Sc enzyme, an RT that is encoded within the second intron of the yeast *COX1* gene (Kennell *et al.*, *Cell 73:*133-146 (1993)). These six motifs, including the invariant aspartic acid residues known to be required for telomerase enzymatic function (Counter *et al.*, *Proc. Natl. Acad. Sci., USA 94:*9202-9207 (1997); Lingner *et al.*, *Science 276:*561-567 (1997)), are found at the appropriate positions of the predicted sequence of hEST2 (Figures 2 and 3A-3F). Thus, the proposed human telomerase catalytic subunit, like its yeast and ciliate counterparts, belongs to the RT superfamily of enzymes.

Although hEST2 shares some sequence similarity with RTs, it is not a conventional RT. Rather, it is far more closely related to the telomerase catalytic subunits of yeast and ciliates than to other RTs. Whereas the BLAST probability of sequence similarity between hEST2 and the telomerase subunits of the single-cell eukaryotes arising by chance is 10^{-13} to 10^{-18}, the chance probability of

sequence similarity with the next most closely related RT, a2-Sc, is 0.12. Beyond the motifs that define the polymerase domains of these various enzymes, hEST2 shows no sequence similarity with RTs. In contrast, in its domains that lie N-terminal to the polymerase domain, BLAST searches identify clear relatedness between hEST2 and both p123 and Est2p, the chance occurrence of these similarities being 1.6×10^{-9} and 1.8×10^{-4} respectively. Many of the sequence identities in the N-termini of hEST2, p123 and Est2p reside in a region just before motif 1 (Figure 2, boxed region). This sequence is not found in RTs, nor is it apparent in other proteins, suggesting the presence of motifs that may be unique to telomerases. Identification of the catalytic subunits of yet other telomerases will be required to validate this possibility.

Even within the hEST2 domain that share sequence similarity with RTs, it is clear that hEST2 is more closely related to the already described telomerases than it is to non-telomerase RTs . For example, the sequence similarity of the region encompassing the RT motifs between hEST2 and the catalytic subunits of yeast and Euplotes has a probability of chance occurrence of 5.7×10^{-6} and 1.9×10^{-5} respectively compared to 0.0056 for a2-Sc, the next most closely related non-telomerase RT. Within the RT motifs are several amino acids that are invariant among the telomerases but divergent between telomerases and non-telomerase RTs, or alternatively nearly invariant among non-telomerase RTs but divergent between these RTs and telomerases (Figures 3A-3F).

In summary, hEST2, Est2p, and p123 form a clearly defined subgroup within the RT family. For these reasons, hEST2 is a human homologue and very likely an orthologue of the microbial enzymes described to date.

Sequencing of a number of cDNA clones has revealed two distinct forms of *hEST2* transcripts. See Figures 5A-5B and Figures 7A-7C. Four independent cDNA clones, isolated from three independently generated libraries deriving from distinct cell types, lack an identical 182 bp segment within the open reading frame. The absence of this segment leads to a shift in reading frame that introduces a premature termination codon. Both forms of the *hEST2* transcript were detected by RT-PCR in a variety of human cell types. Information on the intron-exon boundaries of *hEST2* is not available. The simplest interpretation of these data is that the sequence difference between the two groups of cDNAs reflects the existence of two alternatively spliced mRNAs of the *hEST2* gene. The physiological consequences of the expression of the potential non-functional *hEST2* transcript are obscure at present.

EXAMPLE 3: Chromosomal Localization of *hEST2*

The *hEST2* cDNA was used as a probe in Southern blot analyses of human genomic DNA. These reveal *hEST2* to be a single-copy gene with an estimated size of 40 kb. All the genomic sequences reactive with the cDNA probe appear to be components of this ~40 kb locus, suggesting that there are no other closely related genes in the human genome.

hEST2 was localized to a specific chromosomal region by analyzing two independent panels of hamster-human radiation hybrid (RH) cells with two markers spanning different regions of *hEST2*. Initial mapping using the Genebridge 4 RH panel placed both *hEST2* markers between sequence-tagged sites (STS) WI-9907 and D5S417 (Figure 4). Independent confirmation of this localization was then obtained by mapping carried out with a second panel, the

Stanford G3 RH panel. This second mapping placed both *hEST2* markers next to STS marker AFMA139YA9 (GDB locus D5S678) which itself is localized between the above-mentioned markers WI-9907 and D5S417. These markers are present at the telomeric end of chromosome 5p (Hudson *et al.*, *Science 270:*1945-1954 (1995)).

Mapping of the *hEST2* locus was further refined by localizing it to subband 5p15.33, since the STS markers D5S678 and D5S417 have been assigned to this band on the Genethon YAC contig map (Chumakov *et al.*, *Nature 377:*175-297 (1995)). This localization is consistent with fluorescence in situ hybridization (FISH) analysis of YAC 767E1. This YAC maps further away from the telomere than D5S678 and has been assigned to chromosome 5p15.33 by FISH (Chumakov *et al.*, *Nature 377:*175-297 (1995)).

Chromosome 5p is one of the most common targets for amplification in non-small-cell lung cancers; it is amplified in ~70% of tumors (Balsara *et al.*, *Cancer Res. 56:*645-650 (1997); Petersen *et al.*, *Cancer Res. 57:2331-2335* (1997)). The effects of this amplification on *hEST2* expression levels are unknown.

EXAMPLE 4: Expression of *hEST2* Transcripts

Telomerase becomes activated during tumor progression. and, as discussed above, this activation has been associated with the immortalization of tumor cells. A variety of mechanisms might be invoked to explain such activation, among which is the induction of the expression of one or more telomerase subunits. The telomerase holoenzyme is presumed to exist as a multi-subunit ribonucleoprotein complex and, therefore, the levels of any one of the subunits, including those of the catalytic

subunit described here, might be rate-limiting in determining enzyme activity. Alternatively, the components of the telomerase holoenzyme might be expressed constitutively and subject to various types of post-translational modification that would govern their activity. As mentioned earlier, transcript levels of the telomerase RNA subunit and of the *TP1/TLP1* gene do not necessarily correlate with telomerase activity (Feng *et al.*, *Science 269:*1236-1241 (1995); Avilion *et al.*, *Cancer Res. 56:*645-650 (1996); Blasco *et al.*, *Nat. Genet. 12:*200-204 (1996); Harrington *et al.*, *Science 275:*973-977 (1997); Nakayama *et al.*, *Cell 88:*875-884 (1997)), implying that at least one other mechanism is responsible for regulating human telomerase.

The expression levels of *hEST2* mRNA in various cell types were analyzed, using both RNA Northern hybridizations and RNase protection assays, in order to address one of the remaining possible regulatory mechanisms,. RNA blots prepared from a panel of normal human tissues and human cancer cell lines were probed with cDNA fragments deriving from two independent, non-overlapping regions of the *hEST2* gene. This probing revealed two major RNA species migrating near the 4.4 kb and the 9.5 kb markers, as well as a minor species of ~6 kb (Figures 8A-8F). Each of these RNA species was recognized by both probes, confirming that each represents an *hEST2* mRNA.

hEST2 message was detectable in several normal tissues including thymus, testis, and intestine. Of these, testis (Kim *et al.*, *Science 266:*2011-2015 (1994); Wright *et al.*, *Dev. Genet. 18:*173-179 (1996)) and intestine (Hiyama *et al.*, *Int. J. Oncol. 9:*453-458 (1996)) are known to be

telomerase-positive, while the telomerase status of the thymus has not been reported. In marked contrast, the *hEST2* transcript was undetectable in our assays in most other normal human tissues, including heart, brain, placenta, liver, skeletal muscle, and prostate (Figures 8A 8B, 8D and 8E), all of which have been reported to be telomerase-negative (Kim *et al.*, *Science 266:*2011-2015 (1994); Wright *et al.*, *Dev. Genet. 18:*173-179 (1996); Shay and Bacchetti, *Eur. J. Cancer 33:*787-791 (1997)). The absence of detectable *hEST2* message in ovary may seem paradoxical, as ovary is a germline tissue, and germline tissues have been reported to harbor significant levels of telomerase. However, oocyte division is completed by birth, and while fetal ovary is telomerase-positive (Kim *et al.*, *Science 266:*2011-2015 (1994); Wright *et al.*, *Dev. Genet. 18:*173-179 (1996)), both adult ovarian epithelium and mature oocytes are telomerase-negative (Counter *et al.*, *Proc. Natl. Acad. Sci., USA 91:*2900-2904 (1994); Wright *et al.*, *Dev. Genet. 18:*173-179 (1996)). It is also possible that the levels of *hEST2* are below the threshold of detection. The 7 kb band seen in muscle tissue appears to be an artifact, as it was not observed with an independent *hEST2* probe.

In contrast to its absence in the majority of normal tissues, *hEST2* mRNA was strongly expressed in a variety of cancer cell lines, most strikingly in the leukemic cell lines HL-60 and K-562 (Figures 8C and 8F). It is unclear why only very low levels of *hEST2* message were observed in HeLa cells on this particular Northern blot (Figures 8C and 8F); reanalysis of independently prepared HeLa cell RNA both by Northern blot and by RNase protection demonstrated

that *hEST2* mRNA is present in HeLa cells at high levels comparable to those seen in K-562 cells. The two major and one minor *hEST2* transcript appear to be expressed in the same relative proportions in all cell types that yielded detectable *hEST2* mRNA.

EXAMPLE 5: hEST2 Expression in Primary Human Cancers

The expression of *hEST2* mRNA in cancer cell lines suggested that *hEST2* transcript levels might be elevated in primary tumors as well. To determine if this is the case, RNA was extracted from a variety of tumor samples as well as from normal control tissues and analyzed this for the presence of *hEST2* mRNA using an RNase protection assay. In total, 11 of 11 tumor samples examined showed detectable levels of *hEST2* message (Figures 9A-9D). *hEST2* RNA was undetectable in normal breast and ovarian tissue but was expressed at significant levels in 2 of 2 breast tumors as well as 2 of 2 breast tumor-derived cell lines, and in 4 of 4 ovarian tumors.

As expected from Northern hybridization analysis of *hEST2* RNA (Figures 8A-8F) and the known pattern of telomerase catalytic activity in human tissues (Kim *et al.*, *Science 266*:2011-2015 (1994); Hiyama *et al.*, *Int. J. Oncol. 9*:453-458 (1996)), the *hEST2* transcript was detected at high levels by the RNase protection assay in testis and at moderate levels in colon. Similarly, using the RNase protection assay, four colon tumor samples and a testicular tumor sample were found to express detectable levels of *hEST2* RNA (Figures 9A-9D); two of the colon tumors showed significantly elevated levels as well. These data suggest that the *hEST2* message is expressed in a very high

percentage of tumors and is specifically induced in tumors that arise from telomerase-negative tissues.

EXAMPLE 6: Up-regulation of *hEST2* mRNA Is Associated with Telomerase Activation and Cellular Immortalization

The strong expression of the *hEST2* message observed in several tumors and cancer cell lines suggested that the levels of this transcript are correlated with the amount of telomerase enzyme activity in these various cell types. To investigate this possibility, telomerase activity and *hEST2* RNA level were analyzed in a panel of non-immortalized and immortalized cell lines. Using the TRAP telomerase assay (Kim *et al.*, *Science 266*:2011-2015 (1994)), two mortal fibroblast strains, WI-38 and IMR-90, were found to lack detectable telomerase activity. In contrast, telomerase activity was readily detectable in three immortal cell lines, HeLa, 293, and K-562. The telomerase-negative cells also lacked detectable *hEST2* message as gauged by an RNase protection assay, while the immortal telomerase-positive cell lines expressed significant levels of *hEST2* RNA. These results indicate that *hEST2* message levels correlate closely with telomerase activity.

Thus, as described herein, *hEST2* RNA expression and telomerase activity are present in immortal transformed cells, but absent in mortal normal cells. These findings support the role of induction of *hEST2* expression in the activation of telomerase that occurs during cellular immortalization. That this is the case was shown by analyzing *hEST2* transcript levels by RNase protection, and comparing the levels of hTR and telomerase activity in pre-crisis cells prior to the up-regulation of telomerase, and in post-crisis telomerase-positive immortal cells from

two different transformed human cell populations: Epstein-Barr virus-transformed B lymphocytes (B4 cells) and SV40-T antigen transformed embryonic kidney cells (HA1 cells). Result observed were consistent with previously reported assessments (Counter *et al.*, *EMBO J. 11*:1921-1929 (1992), *Proc. Natl. Acad. Sci., USA 91*:2900-2904 (1994); Avilion *et al.*, *Cancer Res. 56*:645-650 (1996)). Little or no telomerase activity was detected in cells prior to crisis when telomere length decreases, but abundant levels were detected in post-crisis cells which maintain telomere length. In contrast, the levels of *hTR* remained essentially constant throughout the immortalization process. The levels of *hEST2* RNA, on the other hand, parallel telomerase activity during cell immortalization. *hEST2* RNA was undetectable in the pre-crisis cells but was clearly present in the post-crisis, telomerase-positive cells. Induction of *hEST2* message is the rate-limiting step for the activation of telomerase during immortalization.

EXAMPLE 7: Down-regulation of *hEST2* Expression upon Cellular Differentiation

The up-regulation of *hEST2* RNA is associated with the activation of telomerase during cell immortalization. It remained unclear whether conditions that repress telomerase activity in cultured cells might similarly operate by shutting down expression of the *hEST2* mRNA in these cells. Human HL-60 promyelocytic leukemia cells can be induced to differentiate to mature granulocytes by treatment with retinoic acid. During this process, telomerase activity has been shown to decline over a period of two to five days (Sharma *et al.*, *Proc. Natl. Acad. Sci., USA 92*:12343-12346

(1995); Bestilny *et al.*, *Cancer Res. 56:*3796-3802 (1996); Savoysky *et al.*, *Biochem. Biophys. Res. Commun. 226:*329-334 (1996); Xu *et al.*, *Leukemia 10:*1354-1357 (1996)).

Induction of HL-60 differentiation was shown to lead to the disappearance of the *hEST2* mRNA within 24 hours, foreshadowing the loss of telomerase activity which declines more slowly. Once again, this contrasts with the behavior of the human telomerase RNA subunit, whose expression during induced differentiation remains constant, as also previously reported (Bestilny *et al.*, *Cancer Res. 56:*3796-3802 (1996)). Results presented here show that the levels of the *hEST2* mRNA decline precipitously within the first three hours after induced differentiation. This rapid down-modulation of is compatible with a short half-life for the *hEST2* message and suggests that the levels of this RNA species are under tight control in these cells. The contrasting delay in the decline of telomerase activity is consistent with the reported ~24 hour half-life of the enzymatic activity after cycloheximide treatment (Holt *et al.*, *Mol. Cell. Biol. 16:*2932-2939 (1996)).

EXAMPLE 8 Ectopic Expression of hTERT mRNA in Telomerase-Negative Cells and Assessment of Expression on Levels of Telomerase Activity

The *hTERT* cDNA was introduced into a mammalian expression construct carrying the CMV promoter. To distinguish the ectopically expressed hTERT protein from its endogenous counterpart, the C-terminus of the vector-encoded protein was marked with an influenza virus hemagglutinin (HA) epitope tag, yielding the plasmid

pCI-neo-*hTERT-HA*. Such a modification does not affect the catalytic activity of the Est2p protein of *S. cerevisiae* (Counter, C.M., *et al.*, "The catalytic subunit of yeast telomerase", *Proc. Natl. Acad. Sci. USA, 94*:9202-9207 (1997).

This *hTERT-HA* construct and a control empty vector were transfected into cells of the SV40-transformed GM847 human fibroblast line (Pereira-Smith, O.M., and Smith, J.R., "Genetic analysis of indefinite division in human cells: identification of four complementation groups", *Proc. Natl. Acad. Sci. USA, 85*:6042-6046 (1988). In contrast to most immortal human cells, which appear to activate telomerase in order to maintain telomere length (Shay, J.W., and Bacchetti, S., "A survey of telomerase activity in human cancer", *Eur. J. Cancer, 33*:787-791 (1997), the immortal GM847 cells are telomerase-negative (Bryan, T.M., *et al.*, "Telomere elongation in immortal human cells without detectable telomerase activity", *EMBO J., 14*:4240-4248 (1995). Moreover, while the *hTR* gene is transcribed in these cells(Bryan, T.M., *et al.*, "The telomere lengthening mechanism in telomerase-negative immortal human cells does not involve the telomerase RNA subunit", *Hum. Mol. Genet., 6*:921-926 (1997), they lack detectable levels of *hTERT* mRNA (Kilian, A., *et al.*, "Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types", *Hum. Mol. Genet., 6*:2011-2019 (1997). These observations have led to the conclusion that GM847 cells employ a telomerase-independent mechanism to maintain telomere length (Murnane, J.P., *et al.*, "Telomere dynamics in an immortal human cell line", *EMBO J.*,

13:4953-4962 (1994); (Bryan, T.M., *et al.*, "Telomere elongation in immortal human cells without detectable telomerase activity", *EMBO J.*, *14*:4240-4248 (1995), perhaps analogous to the recombination-based pathway used by yeast cells to allow chromosomes to maintain telomeres in the absence of a functional telomerase pathway (Lundblad, V., and Blackburn, E.H., "An alternative pathway for yeast telomere maintenance rescues est1- senescence. Cell 73, 347-360 (1993). Unlike normal, telomerase-negative human somatic cells, which lack replicative immortality, these GM847 cells could be propagated indefinitely following transfection, allowing us to study the properties of clonally isolated cell populations that have stably acquired the introduced *hTERT* gene.

A number of stably transfected GM847 cell clones were generated with either the control vector or the *hTERT-HA* expression vector, mRNA expression of *hTERT* was analyzed by RNase protection using probes that specifically recognize either the transfected *hTERT-HA* mRNA or the endogenous *hTERT* transcript. As expected, *hTERT-HA* transcript was detected only in GM847 sublines stably transfected with an *hTERT-HA* expression plasmid, but not in untransfected telomerase-positive control cell lines 293 and HL-60, in the parental GM847 line or in GM847 sublines transfected with the empty vector. The cells expressing *hTERT-HA* did not express the endogenous *hTERT* transcript, despite the fact that this RNA is clearly detected in telomerase-positive cells. Lastly, in accord with previous observations that the level of the *hTR RNA* subunit of telomerase does not correlate with enzyme activity, this RNA was detected in all cells tested, irrespective of whether the cells had telomerase activity. An actin

control probe demonstrates comparable loading of RNA from each cell line. Moreover, the specificity of the probes used was demonstrated by their failure to protect tRNA.

Expression of hTERT-HA was also analysed at the protein level, by immunoblotting with an anti-HA antibody probe directed against the HA tag of the vector-encoded hTERT protein. A ~ 130 kDa product corresponding to the predicted size of hTERT was detected in those lines derived from GM847 cells that were stably transfected with the hTERT-HA expression construct, but not in those cell clones that had been transfected with the empty vector. The antibody likewise did not detect endogenous (untagged) hTERT known to be expressed in 293 cells (Meyerson, M., *et al.* "*hEST2*, the Putative Human Telomerase Catalytic Subunit Gene, Is Up-Regulated in Tumor Cells and during Immortalization," *Cell*, *90*:785-795 (1997), a telomerase-positive control cell line.

Assessment of whether these hitherto telomerase-negative GM847 cell lines acquired telomerase activity together with the stable ectopic expression of *hTERT* was carried out. Telomerase activity was measured in these different cell lines by assaying the ability of a cellular extract to elongate a primer in a telomerase-specific manner. The products of this *in vitro* reaction are subsequently detected by specific PCR amplification, yielding a ladder of products differing from one another by 6 bp (Kim, N.W., *et al.*, "Specific association of human telomerase activity with immortal cells and cancer", *Science*, *266*:2011-2015 (1994)). Telomerase activity was detected in 293 cells, a cell line with one of the highest levels of telomerase activity known. This activity was sensitive to heat treatment of the extract, which inactivates telomerase (Kim, N.W., *et al.*, "Specific

association of human telomerase activity with immortal cells and cancer", *Science*, *266*:2011-2015 (1994)). In contrast, almost no telomerase products were detectable following assay of extracts from untransfected GM847 cells or from GM847 sublines stably transfected with the empty control vector. This inability to detect telomerase was not due to the presence of a PCR-inhibiting activity, as an internal control was specifically PCR-amplified, (Kim, N.W., and Wu, F. "Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP)", *Nucleic Acids Res.*, *25*: 2595-2597 (1997).

In marked contrast, telomerase activity was readily detectable in those clones of GM847 cells that were stably transfected with the *hTERT-HA* expression vector and this activity was sensitive to heat inactivation. The levels of telomerase activity observed in these transfectants approached those seen in extracts from 293 cells. The restoration of telomerase activity in the cells transfected with *hTERT-HA* was not due to the up-regulation of the endogenous *hTERT* gene, as evidenced by failure to detect the corresponding mRNA in these cells. Thus, ectopic expression of *hTERT* in previously telomerase-negative cells is sufficient to generate telomerase activity at levels comparable to those found in immortalized telomerase-positive cells.

The telomerase activity detected in cells transfected with the *hTERT-HA* expression vector was physically associated with ectopically produced hTERT-HA, confirming that hTERT is, as predicted, a constituent of the telomerase holoenzyme. Telomerase activity could be immunoprecipitated with an anti-HA monoclonal antibody from

extracts of either 293 cells or GM847 cells that ectopically express *hTERT-HA*. The telomerase activity was not immunoprecipitated with an antibody directed against an irrelevant antigen (anti-p53), nor was it immunoprecipitated when either antibody was incubated with extracts prepared from control vector-transfected GM847 cells. Taken together, these data indicate that telomerase activity is specifically co-immunoprecipitated with hTERT-HA.

Whether the observed induction of telomerase activity following ectopic hTERT expression was unique to GM847 cells was also determined. This was done by transiently transfecting WI-38 normal human fibroblasts, which lack detectable levels of telomerase activity and *hTERT* message but express the hTR gene (Meyerson, M., *et al.*, "*hEST2*, the Putative Human Telomerase Catalytic Subunit Gene, Is Up-Regulated in Tumor Cells and during Immortalization. Cell *90*, 785-795 (1997), with the *hTERT-HA* expression construct. Since transient transfection of normal human cells is extremely inefficient, the plasmid pGreenLantern-1, which encodes the green fluorescent protein (GFP), was co-transfected with either the *hTERT-HA* expression vector or the control vector. Cells expressing GFP, and hence quite likely the co-transfected plasmid, were sorted by virtue of their fluorescence.

Extracts derived from both populations of fluorescing cells were assayed for telomerase activity. Whereas fibroblasts transfected with pGreenLantern-1 and pCI-neo vector alone lacked enzymatic activity, those co-transfected with the *hTERT-HA* expression vector were clearly telomerase-positive. Transfection with pCI-neo-*hTERT-HA* of IMR-90 cells, another telomerase-

negative normal human fibroblast cell strain that does not normally express *hTERT* (Kim, N.W., *et al.*, "Specific association of human telomerase activity with immortal cells and cancer", *Science, 266:* 2011-2015 (1994); (Meyerson, M., *et al.*,"*hEST2*, the Putative Human Telomerase Catalytic Subunit Gene, Is Up-Regulated in Tumor Cells and during Immortalization", *Cell, 90:* 785-795 (1997), also gave rise to telomerase activity (not shown). Thus, ectopic expression of *hTERT* in these two types of normal human somatic cells results in readily detectable telomerase activity.

Taken together, these data demonstrate that the ectopic expression of hTERT in otherwise telomerase-negative human cells is both necessary and sufficient for induction of telomerase activity. Furthermore, the physical association of hTERT with telomerase activity confirms that hTERT is a telomerase subunit. The fact that forced expression of hTERT sufficed to impart telomerase activity indicates that levels of *hTR* mRNA and TP-1 or other still unidentified components of the telomerase holoenzyme are not rate-limiting determinants of telomerase activity in these cells. Up-regulation of the *hTERT* gene is therefore the sole barrier to activation of telomerase in the tested cells.

It still remains to be determined if telomerase activity can be restored in this fashion in all telomerase-negative cells. However, results described here show that activity is conferred by ectopic expression of hTERT in cell types representative of two known classes of telomerase-negative cells: telomerase-negative immortal cell lines and normal mortal human cell strains. Thus, it is likely that expression of *hTERT* mRNA is the

rate-determining step for telomerase activation in other human cells lacking enzyme activity. This makes the regulation of transcription from the *hTERT* promoter a potential target for modulation during tumorigenesis and cell immortalization.

Experimental Procedures

The following methods and materials were used in the experiments which are described above.

Radiation Hybrid Mapping

25 ng of genomic DNA from hybrid clones of the Genebridge 4 and Stanford G3 radiation hybrid (RH) mapping panels (Research Genetics, Inc. Huntsville, AL) was PCR-amplified with the primer pair M1 (forward-5' -CACAGCCAGGCCGAGAGCAGA- 3'(SEQ ID NO.: 48) and reverse-5' -AGGCCTCAGCCGGACACTCAG- 3')(SEQ ID NO.: 49), yielding a 170 bp fragment in the 3'-untranslated region, and with primer pair M2 (forward-5' -GAAGAAAACATTTCTGTCGTG- 3'(SEQ ID NO.: 50) and reverse- 5' -GCCCTTGGCCCCCAGCGACAT- 3')(SEQ ID NO.: 51), generating a 180 bp fragment crossing a putative intron-exon boundary near the *hEST2* stop codon. PCR was carried out for 35 cycles of 94°C for 0.5 min., 69°C for M1 or 65°C for M2 for 0.5 min. and 72°C for 1.5 min. The results of the PCR screening were analyzed using the statistical program RHMAP provided through the following two e-mail servers on the World Wide Web: http://shgc-www.stanford.edu and http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl. Linkage of STS markers to a physical chromosomal map was achieved by accessing the servers: http://www.cephb.fr/ceph-genethon-map.html and http://www-genome.wi.mit.edu. Comparison to YACs

physically mapped by FISH was achieved with the server: ftp://ftp.cephb.fr/pub/ceph-genethon-map/FISH/29MAR95.DAT.

RNA Isolation from Cell Lines and Primary Tissues

Cell lines were obtained from the American Type Culture Collection and grown under standard conditions. For HL-60 differentiation assays, cells were pelleted and resuspended in normal growth medium plus all-*trans* retinoic acid (Sigma) at a final concentration of 1 μM.

All primary normal and tumor tissues were obtained from the Massachusetts General Hospital tumor bank. Tissues were processed in a tissue homogenizer. RNA samples were prepared in the RNA Stat-60 solution (Tel-Test "B", Friendswood, Texas) according to the manufacturer's protocol.

Northern Hybridization

Duplicate filters containing poly A(+)-selected mRNAs from various human tissues and cell lines (Multiple Tissue Northern Blots, Clontech, Palo Alto, CA), were incubated according to the manufacturer's instructions with two independent *hEST2* probes. One probe was derived by PCR-amplifying plasmid 712562 with primers HT-1 and HT-5 to generate the 377 bp fragment described above; the second, more 3' probe was generated from the 1064 bp *Stu* I fragment of the same plasmid. The duplicate blots were then rehybridized with a β-actin probe. Northern blots for detection of *hTR* were performed with total RNA and an *hTR*-specific probe (Feng *et al.*, *Science 269:*1236-1241 (1995)).

RNAse Protection Analysis

Radiolabelled RNA probes for RNAse protection analysis were synthesized using [α-^{32}P]UTP, T7 RNA polymerase and

the MAXIscript kit (Ambion, Austin, TX). DNA templates for probe synthesis were created as follows. For the *hEST2* probe, the insert from plasmid clone 712562 was subcloned into pUHD 10-3 (a gift from M. Gossen), and template DNA was synthesized by PCR using a forward plasmid-specific primer and a reverse primer containing 18bp of *hEST2* complementary sequence (5'-TCTCTGCGGAAGTTCTG)(SEQ ID NO.: 52) and the T7 promoter sequence. For the β-actin probe, template DNA was synthesized by PCR on a human β-actin cDNA insert (Clontech). The *hEST2* and the β-actin control probes were hybridized in the same reaction tube. The *hTR* probe was synthesized directly from the linearized pGRN83 plasmid (Feng *et al.*, *Science 269:*1236-1241 (1995)).

RNase protection analysis was performed using the HybSpeed RPA kit (Ambion) according to the manufacturer's protocol. Briefly, sample RNA and radiolabelled RNA probes were coprecipitated in ethanol, resuspended in hybridization buffer, hybridized at 68° C, then digested with RNases A and T1. Samples were then re-precipitated and analyzed on a 6% denaturing polyacrylamide gel.

Telomerase assays

Telomerase repeat amplification protocol (TRAP) assays were performed as described (Kim *et al.*, *Science 266:*2011-2015 (1994)), in some cases using the TRAPeze telomerase detection kit (Oncor, Gaithersburg, MD).

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the

invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

CLAIMS

What is claimed is:

1. Isolated DNA encoding the catalytic subunit of a eukaryotic telomerase holoenzyme.

2. Isolated DNA of Claim 1 which encodes the catalytic subunit of a yeast telomerase holoenzyme.

3. Isolated DNA of Claim 2 wherein the DNA is selected from the group consisting of: DNA of SEQ ID NO.: 1; DNA which hybridizes to the complement of SEQ ID NO.: 1; and DNA which encodes the amino acid sequence of SEQ ID NO.: 2.

4. Isolated DNA of Claim 1 which encodes the catalytic subunit of a human telomerase holoenzyme.

5. Isolated DNA of Claim 4 wherein the DNA is selected from the group consisting of: DNA comprising the nucleotide sequence of SEQ ID NO.: 35; DNA which encodes hEST2 protein having the amino acid sequence of SEQ ID NO.: 3; and DNA comprising a nucleotide sequence which hybridizes to the complement of SEQ ID NO.: 35.

6. Isolated DNA of Claim 4 which is an isolated gene which localizes to human chromosome subband 5p15.33.

7. Isolated DNA comprising DNA encoding a human telomerase catalytic subunit, wherein the DNA encodes an RNA transcript which is up-regulated in a human tumor.

8. Isolated DNA of Claim 7 which is an isolated human gene which localizes to human chromosome subband 5p15.33.

9. Isolated DNA of Claim 7 which comprises *hEST2* cDNA of SEQ ID NO.: 35 or DNA which hybridizes to the complement of *hEST2* cDNA under conditions of high stringency.

10. Isolated DNA of Claim 7 wherein the human tumor is selected from the group consisting of: breast tumors, ovarian tumors, colon tumors and testicular tumors.

11. Isolated DNA of Claim 7 which encodes a human telomerase catalytic subunit which comprises approximately 1132 amino acid residues.

12. Isolated DNA of Claim 8 wherein the RNA transcript is also up-regulated in a cancer cell line, a telomerase-positive tissue or both.

13. Isolated mRNA which encodes a human telomerase catalytic subunit, wherein expression of the mRNA is up-regulated in a human tumor.

14. Isolated mRNA of Claim 13 wherein the human tumor is selected from the group consisting of: breast tumors, ovarian tumors, colon tumors and testicular tumors.

15. Isolated mRNA of Claim 13 wherein the mRNA has the sequence of SEQ ID NO.: 36.

16. A nucleic acid probe comprising DNA unique to *hEST2* DNA, *EST2* DNA and p123 DNA.

17. The nucleic acid probe of Claim 16 comprising DNA which encodes the telomerase motif of hEST2 protein.

18. The nucleic acid probe of Claim 17 comprising nucleotides which encode amino acid residues 556 to 565 of SEQ ID NO.: 3 or nucleotides which encode amino acid residues 1 to 50 of hEST2 protein or a portion thereof.

19. A method of assessing cells for malignancy or an increased likelihood of progression to malignancy, comprising:

a) obtaining cells to be assessed from an individual;

b) processing cells obtained in a) to render DNA and/or RNA in the cells available for annealing of the DNA and/or RNA with complementary poly- or oligonucleotides, thereby producing processed cells;

c) combining processed cells with hEST2 DNA or RNA under conditions appropriate for annealing of DNA and/or RNA with complementary poly- or oligonucleotides, thereby producing a combination,

wherein if annealing occurs in the combination produced in c), it is indicative of activation of telomerase and malignancy or increased likelihood of progression to malignancy.

20. The method of Claim 21 wherein the poly- or oligonucleotides are DNA which encodes the telomerase

motif or amino acid residues 1 to 50 of SEQ ID NO.: 3 or a portion thereof.

21. A method of diagnosing or aiding in the diagnosis of development of malignancy in an individual, comprising:

a) obtaining cells from the individual;

b) processing the cells obtained in a) to render proteins in the cells available for binding with antibodies;

c) combining the product of b) with antibodies which bind hEST2 protein; and

d) determining whether binding occurs between the antibodies which bind hEST2 protein and protein in the cell,

wherein if binding occurs, it is indicative of the presence of hEST2 protein in the cells and of malignancy or an increased likelihood of development of malignancy in the individual.

22. The method of Claim 21 wherein the antibodies which bind hEST2 protein bind the telomerase motif of hEST2 protein or bind amino acid residues 1 to 50 of SEQ ID NO.: 3 or a portion thereof.

23. A method of reducing expression of *hEST2* RNA and hEST2 protein in cells of an individual, comprising administering to the individual a drug selected from the group consisting of: drugs which inhibit or bind *hEST2* RNA and prevent or reduce production of hEST2 protein and drugs which inhibit hEST2 protein function or activity.

24. The method of Claim 23 wherein the drug is selected from the group consisting of: small organic molecules; enzymes which degrade hEST2 protein; enzyme inhibitors; HEST2 transcriptional regulators; antisense molecules and dominant negative forms of hEST2 protein.

25. A method of treating cancer in an individual, comprising administering to the individual a drug which inhibits or binds *hEST2* RNA and prevents or reduces production of hEST2 protein or a drug which inhibits hEST2 protein function or activity, under conditions appropriate for the drug to enter cells of the individual, whereby the drug inhibits or binds *hEST2* RNA and prevents or reduces production of hEST2 protein or inhibits hEST2 protein function or activity.

26. The method of Claim 25 wherein the drug is selected from the group consisting of: enzymes which degrade hEST2 protein; enzyme inhibitors; hEST2 transcriptional regulators; antisense molecules and dominant negative forms of hEST2 protein.

27. A method of increasing lifespan of cells, comprising:
(a) introducing into the cells *hEST2* DNA, under conditions appropriate for expression of *hEST2* DNA, whereby hEST2 protein is expressed or
(b) introducing hEST2 protein into the cells, whereby sufficient hEST2 protein is expressed or present in the cells to increase lifespan of the cells.

28. The method of Claim 27, wherein the cells are cells in culture.

29. A method of decreasing lifespan of cells in an individual by reducing hEST2 protein function or activity, comprising administering to the individual a drug selected from the group consisting of:
(a) drugs which inhibit or bind *hEST2* RNA and prevent or reduce production of hEST2 protein;
(b) drugs which inhibit hEST2 protein function or activity; and
(c) drugs which inhibit or bind *hEST2* DNA and prevent or reduce production of *hEST2* RNA transcript,
wherein the drug is administered to the individual under conditions appropriate for entry of the drug into cells in sufficient quantity to reduce hEST2 protein function or activity.

30. The method of Claim 29 wherein the drug is selected from the group consisting of: small organic molecules; enzymes which degrade hEST2 protein; enzyme inhibitors; HEST2 transcriptional regulators; antisense molecules and dominant negative forms of hEST2 protein.

```
ATGAAAATCTTATTCGAGTTCATTCAAGACAAGCTTGACATTGATCTACAGACCAACAGTACTTACAAAGAAAATTTAAAATGTGGTCACTTCAATGGCC
 M  K  I  L  F  E  F  I  Q  D  K  L  D  I  D  L  Q  T  N  S  T  Y  K  E  N  L  K  C  G  H  F  N  G
TCGATGAAATTCTAACTACGTGTTTCGCACTACCAAATTCAAGAAAAATAGCATTACCATGCCTTCCTGGTGACTTAAGCCACAAAGCAGTCATTGATCA
L  D  E  I  L  T  T  C  F  A  L  P  N  S  R  K  I  A  L  P  C  L  P  G  D  L  S  H  K  A  V  I  D  H
CTGCATCATTTACCTGTTGACGGGCGAATTATACAACAACGTACTAACATTTGGCTATAAAATAGCTAGAAATGAAGATGTCAACAATAGTCTTTTTTGC
  C  I  I  Y  L  L  T  G  E  L  Y  N  N  V  L  T  F  G  Y  K  I  A  R  N  E  D  V  N  N  S  L  F  C
CATTCTGCAAATGTTAACGTTACGTTACTGAAAGGCGCTGCTTGGAAAATGTTCCACAGTTTGGTCGGTACATACGCATTCGTTGATTTATTGATCAATT
 H  S  A  N  V  N  V  T  L  L  K  G  A  A  W  K  M  F  H  S  L  V  G  T  Y  A  F  V  D  L  L  I  N
ATACAGTAATTCAATTTAATGGGCAGTTTTTCACTCAAATCGTGGGTAACAGATGTAACGAACCTCATCTGCCGCCCAAATGGGTCCAACGATCATCCTC
Y  T  V  I  Q  F  N  G  Q  F  F  T  Q  I  V  G  N  R  C  N  E  P  H  L  P  P  K  W  V  Q  R  S  S  S
ATCATCCGCAACTGCTGCGCAAATCAAACAACTTACAGAACCAGTGACAAATAAACAATTCTTACACAAGCTCAATATAAATTCCTCTTCTTTTTTTCCT
  S  S  A  T  A  A  Q  I  K  Q  L  T  E  P  V  T  N  K  Q  F  L  H  K  L  N  I  N  S  S  S  F  F  P
TATAGCAAGATCCTTCCTTCATCATCATCTATCAAAAAGCTAACTGACTTGAGAGAAGCTATTTTTCCCACAAATTTGGTTAAAATTCCTCAGAGACTAA
 Y  S  K  I  L  P  S  S  S  S  I  K  K  L  T  D  L  R  E  A  I  F  P  T  N  L  V  K  I  P  Q  R  L
AGGTACGAATTAATTTGACGCTGCAAAAGCTATTAAAGAGACATAAGCGTTTGAATTACGTTTCTATTTTGAATAGTATTTGCCCACCATTGGAAGGGAC
K  V  R  I  N  L  T  L  Q  K  L  L  K  R  H  K  R  L  N  Y  V  S  I  L  N  S  I  C  P  P  L  E  G  T
CGTATTGGACTTGTCGCATTTGAGTAGGCAATCACCAAAGGAACGAGTCTTGAAATTTATCATTGTTATTTTACAGAAGTTATTACCCCAAGAAATGTTT
  V  L  D  L  S  H  L  S  R  Q  S  P  K  E  R  V  L  K  F  I  I  V  I  L  Q  K  L  L  P  Q  E  M  F
GGCTCAAAGAAAAATAAAGGAAAAATTATCAAGAATCTAAATCTTTTATTAAGTTTACCCTTAAATGGCTATTTACCATTTGATAGTTTGTTGAAAAAGT
 G  S  K  K  N  K  G  K  I  I  K  N  L  N  L  L  L  S  L  P  L  N  G  Y  L  P  F  D  S  L  L  K  K
TAAGATTAAAGGATTTTCGGTGGTTGTTCATTTCTGATATTTGGTTCACCAAGCACAATTTTGAAAACTTGAATCAATTGGCGATTTGTTTCATTTCCTG
L  R  L  K  D  F  R  W  L  F  I  S  D  I  W  F  T  K  H  N  F  E  N  L  N  Q  L  A  I  C  F  I  S  W
GCTATTTAGACAACTAATTCCCAAAATTATACAGACTTTTTTTTACTGCACCGAAATATCTTCTACAGTGACAATTGTTTACTTTAGACATGATACTTGG
  L  F  R  Q  L  I  P  K  I  I  Q  T  F  F  Y  C  T  E  I  S  S  T  V  T  I  V  Y  F  R  H  D  T  W
AATAAACTTATCACCCCTTTTATCGTAGAATATTTTAAGACGTACTTAGTCGAAAACAACGTATGTAGAAACCATAATAGTTACACGTTGTCCAATTTCA
 N  K  L  I  T  P  F  I  V  E  Y  F  K  T  Y  L  V  E  N  N  V  C  R  N  H  N  S  Y  T  L  S  N  F
ATCATAGCAAAATGAGGATTATACCAAAAAAAAGTAATAATGAGTTCAGGATTATTGCCATCCCATGCAGAGGGGCAGACGAAGAAGAATTCACAATTTA
N  H  S  K  M  R  I  I  P  K  K  S  N  N  E  F  R  I  I  A  I  P  C  R  G  A  D  E  E  E  F  T  I  Y
TAAGGAGAATCACAAAAATGCTATCCAGCCCACTCAAAAAATTTTAGAATACCTAAGAAACAAAAGGCCGACTAGTTTTACTAAAATATATTCTCCAACG
  K  E  N  H  K  N  A  I  Q  P  T  Q  K  I  L  E  Y  L  R  N  K  R  P  T  S  F  T  K  I  Y  S  P  T
CAAATAGCTGACCGTATCAAAGAATTTAAGCAGAGACTTTTAAAGAAATTTAATAATGTCTTACCAGAGCTTTATTTCATGAAATTTGATGTCAAATCTT
 Q  I  A  D  R  I  K  E  F  K  Q  R  L  L  K  K  F  N  N  V  L  P  E  L  Y  F  M  K  F  D  V  K  S
GCTATGATTCCATACCAAGGATGGAATGTATGAGGATACTCAAGGATGCGCTAAAAAATGAAAATGGGTTTTTCGTTAGATCTCAATATTTCTTCAATAC
C  Y  D  S  I  P  R  M  E  C  M  R  I  L  K  D  A  L  K  N  E  N  G  F  F  V  R  S  Q  Y  F  F  N  T
CAATACAGGTGTATTGAAGTTATTTAATGTTGTTAACGCTAGCAGAGTACCAAAACCTTATGAGCTATACATAGATAATGTGAGGACGGTTCATTTATCA
 N  T  G  V  L  K  L  F  N  V  V  N  A  S  R  V  P  K  P  Y  E  L  Y  I  D  N  V  R  T  V  H  L  S
AATCAGGATGTTATAAACGTTGTAGAGATGGAAATATTTAAAACAGCTTTGTGGGTTGAAGATAAGTGCTACATTAGAGAAGATGGTCTTTTTCAGGGCT
 N  Q  D  V  I  N  V  V  E  M  E  I  F  K  T  A  L  W  V  E  D  K  C  Y  I  R  E  D  G  L  F  Q  G
```

FIG. 1A

```
CTAGTTTATCTGCTCCGATCGTTGATTTGGTGTATGACGATCTTCTGGAGTTTTATAGCGAGTTTAAAGCCAGTCCTAGCCAGGACACATTAATTTTAAA
S  S  L  S  A  P  I  V  D  L  V  Y  D  D  L  L  E  F  Y  S  E  F  K  A  S  P  S  Q  D  T  L  I  L  K
ACTGGCTGACGATTTCCTTATAATATCAACAGACCAACAGCAAGTGATCAATATCAAAAAGCTTGCCATGGGCGGATTTCAAAAATATAATGCGAAAGCC
  L  A  D  D  F  L  I  I  S  T  D  Q  Q  Q  V  I  N  I  K  K  L  A  M  G  G  F  Q  K  Y  N  A  K  A
AATAGAGACAAAATTTAGCCGTAAGCTCCCAATCAGATGATACGGTTATTCAATTTTGTGCAATGCACATATTTGTTAAAGAATTGGAAGTTTGGA
  N  R  D  K  I  L  A  V  S  S  S  Q  S  D  D  D  T  V  I  Q  F  C  A  M  H  I  F  V  K  E  L  E  V  W
AACATTCAAGCACAATGAATTAATTTCCATATCCGTTCGAAATCTAGTAAAGGGATATTTCGAAGTTTAATAGCGCTGTTTAACACTAGAATCTCTTATAA
K  H  S  S  T  M  N  N  F  H  I  R  S  K  S  S  K  G  I  F  R  S  L  I  A  L  F  N  T  R  I  S  Y  K
AACAATTGACACAAATTTAAATTCAACAAACACCGTTCTCATGCAAATTGATCATGTTGTAAAGAACATTTCGGAATGTTATAAATCTGCTTTTAAGGAT
  T  I  D  T  N  L  N  S  T  N  T  V  L  M  Q  I  D  H  V  V  K  N  I  S  E  C  Y  K  S  A  F  K  D
CTATCAATTAATGTTACGCAAAATATGCAATTTCATTCGTTCTTACAACGCATCATTGAAATGACAGTCAGCGGTTGTCCAATTACGAAATGTGATCCTT
  L  S  I  N  V  T  Q  N  M  Q  F  H  S  F  L  Q  R  I  I  E  M  T  V  S  G  C  P  I  T  K  C  D  P
TAATCGAGTATGAGGTACGATTCACCATATTGAATGGATTTTTGGAAAGCCTATCTTCAAACACATCAAAATTTAAAGATATATCATTCTTTTGAGAAA
L  I  E  Y  E  V  R  F  T  I  L  N  G  F  L  E  S  L  S  S  N  T  S  K  F  K  D  N  I  I  L  L  R  K
GGAAATTCAACACTTGCAAGCATATATATATATATACATATAGTTAATTAG  2655
  E  I  Q  H  L  Q  A  Y  I  Y  I  Y  I  H  I  V  N
```

FIG. 1B

FIG. 2A

motif 1
motif 2
motif 3
motif 4
motif 5
motif 6
hEST2
p123
Est2p

FIG. 2B

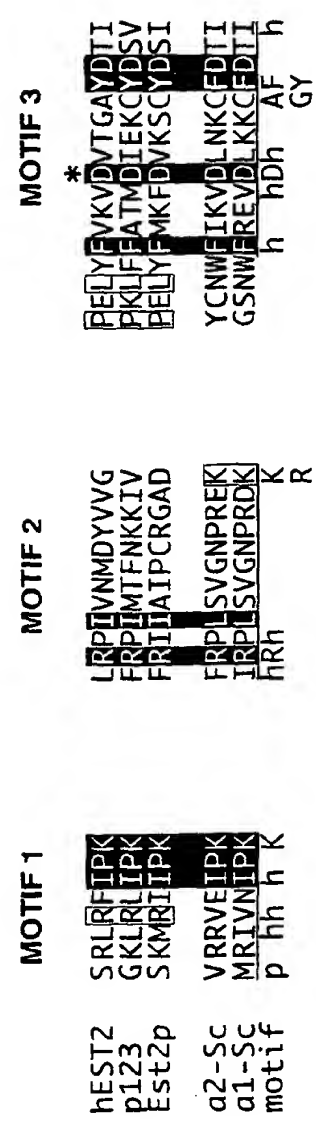
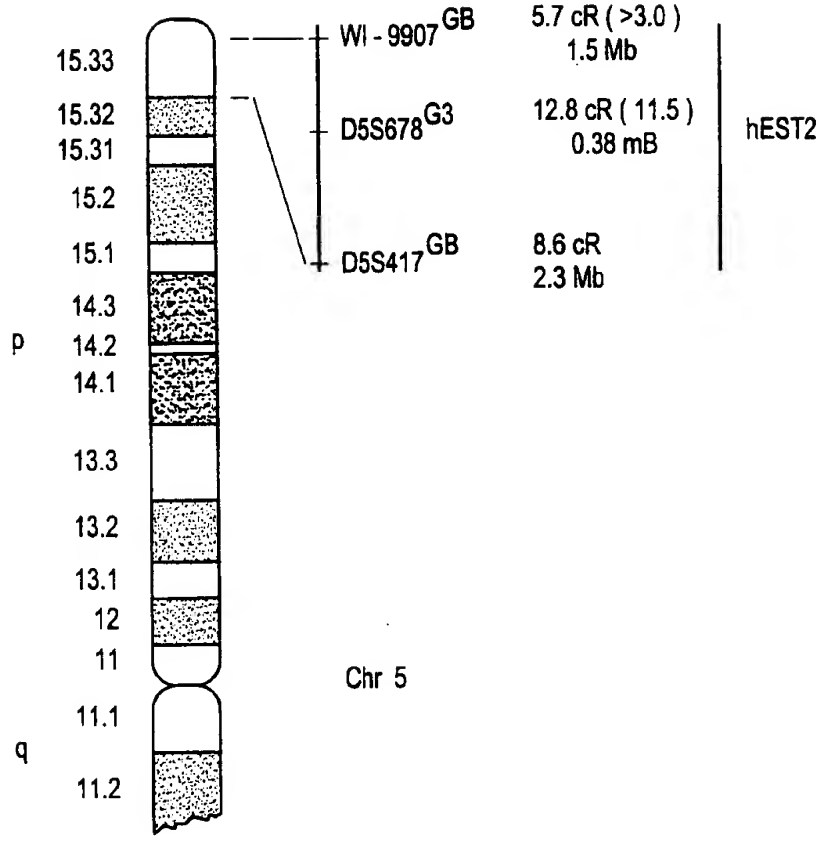


FIG. 3A FIG. 3B FIG. 3C

FIG. 3D FIG. 3E FIG. 3F

FIG. 4


WI - 9907GB
5.7 cR (>3.0)
1.5 Mb
D5S678G3
12.8 cR (11.5)
0.38 mB
hEST2
D5S417GB
8.6 cR
2.3 Mb
15.33
15.32
15.31
15.2
15.1
14.3
p
14.2
14.1
13.3
13.2
13.1
12
11
11.1
q
11.2
Chr 5

```
        10         20         30         40         50         60         70         80
CAGGCAGCGCTGCGTCCTGCTGCGCACGTGGGAAGCCCTGGCCCCGGCCACCCCCGCGATGCCGCGCGCTCCCCGCTGCC   80
GAGCCGTGCGCTCCCTGCTGCGCAGCCACTACCGCGAGGTGCTGCCGCTGGCCACGTTCGTGCGGCGCCTGGGGCCCCAG  160
GGCTGGCGGCTGGTGCAGCGCGGGGACCCGGCGGCTTTCCGCGCGCTGGTGGCCCAGTGCCTGGTGTGCGTGCCCTGGGA  240
CGCACGGCCGCCCCCCCGCCGCCCCCTCCTTCCGCCAGGTGTCCTGCCTGAAGGAGCTGGTGGCCCGAGTGCTGCAGAGGC  320
TGTGCGAGCGCGGCGCGAAGAACGTGCTGGCCTTCGGCTTCGCGCTGCTGGACGGGGCCCGCGGGGGCCCCCCCCGAGGCC  400

        410        420        430        440        450        460        470        480
TTCACCACCAGCGTGCGCAGCTACCTGCCCAACACGGTGACCGACGCACTGCGGGGGAGCGGGGCGTGGGGGCTGCTGTT   480
GCGCCGCGTGGGCGACGACGTGCTGGTTCACCTGCTGGCACGCTGCGCGCTCTTTGTGCTGGTGGCTCCCAGCTGCGCCT   560
ACCAGGTGTGCGGGCCGCCGCTGTACCAGCTCGGCGCTGCCACTCAGGCCCGGCCCCCGCCACACGCTAGTGGACCCCGA   640
AGGCGTCTGGGATGCGAACGGGCCTGGAACCATAGCGTCAGGGAGGCCGGGGTCCCCCTGGGCCTGCCAGCCCCGGGTGC   720
GAGGAGGCGCGGGGGCAGTGCCAGCCGAAGTCTGCCGTTGCCCAAGAGGCCCAGGCGTGGCGCTGCCCCTGAGCCGGAGC   800

        810        820        830        840        850        860        870        880
GGACGCCCGTTGGGCAGGGGTCCTGGGCCCACCCGGGCAGGACGCGTGGACCGAGTGACCGTGGTTTCTGTGTGGTGTCA   880
CCTGCCAGACCCGCCGAAGAAGCCACCTCTTTGGAGGGTGCGCTCTCTGGCACGCGCCACTCCCACCCATCCGTGGGCCG   960
CCAGCACCACGCGGGCCCCCCATCCACATCGCGGCCACCACGTCCCTGGGACACGCCTTGTCCCCCGGTGTACGCCGAGA  1040
CCAAGCACTTCCTCTACTCCTCAGGCGACAAGGAGCAGCTGCGGCCCTCCTTCCTACTCAGCTCTCTGAGGCCCAGCCTG  1120
ACTGGCGCTCGGAGGCTCGTGGAGACCATCTTTCTGGGTTCCAGGCCCTGGATGCCAGGGACTCCCCGCAGGTTGCCCCG  1200

        1210       1220       1230       1240       1250       1260       1270       1280
CCTGCCCCAGCGCTACTGGCAAATGCGGCCCCTGTTTCTGGAGCTGCTTGGGAACCACGCGCAGTGCCCCTACGGGGTGC  1280
TCCTCAAGACGCACTGCCCGCTGCGAGCTGCGGTCACCCCAGCAGCCGGTGTCTGTGCCCGGGAGAAGCCCCAGGGCTCT  1360
GTGGCGGCCCCCGAGGAGGAGGACACAGACCCCCGTCGCCTGGTGCAGCTGCTCCGCCAGCACAGCAGCCCCTGGCAGGT  1440
GTACGGCTTCGTGCGGGCCTGCCTGCGCCGGCTGGTGCCCCCAGGCCTCTGGGGCTCCAGGCACAACGAACGCCGCTTCC  1520
TCAGGAACACCAAGAAGTTCATCTCCCTGGGGAAGCATGCCAAGCTCTCGCTGCAGGAGCTGACGTGGAAGATGAGCGTG  1600

        1610       1620       1630       1640       1650       1660       1670       1680
CGGGGCTGCGCTTGGCTGCGCAGGAGCCCAGGGGTTGGCTGTGTTCCGGCCGCAGAGCACCGTCTGCGTGAGGAGATCCT  1680
GGCCAAGTTCCTGCACTGGCTGATGAGTGTGTACGTCGTCGAGCTGCTCAGGTCTTTCTTTATGTCACGGAGACCACGT  1760
TTCAAAAGAACAGGCTCTTTTTCTACCGGAAGAGTGTCTGGAGCAAGTTGCAAAGCATTGGAATCAGACAGCACTTGAAG  1840
AGGGTGCAGCTGCGGGAGCTGTCGGAAGCAGAGGTCAGGCAGCATCGGGAAGCCAGGCCCGCCCTGCTGACGTCCAGACT  1920
CCGCTTCATCCCCAAGCCTGACGGGCTGCGGCCGATTGTGAACATGGACTACGTCGTGGGAGCCAGAACGTTCCGCAGAG  2000

        2010       2020       2030       2040       2050       2060       2070       2080
AAAAGAGGGCCGAGCGTCTCACCTCGAGGGTGAAGGCACTGTTCAGCGTGCTCAACTACGAGCGGGCGCGGCGCCCCGGC  2080
CTCCTGGGCGCCTCTGTGCTGGGCCTGGACGATATCCACAGGGCCTGGCGCACCTTCGTGCTGCGTGTGCGGGCCCAGGA  2160
CCCGCCGCCTGAGCTGTACTTTGTCAAGGTGGATGTGACGGGCGCGTACGACACCATCCCCCAGGACAGGCTCACGGAGG  2240
TCATCGCCAGCATCATCAAACCCCAGAACACGTACTGCGTGCGTCGGTATGCCGTGGTCCAGAAGGCCGCCCATGGGCAC  2320
GTCCGCAAGGCCTTCAAGAGCCACGTCTCTACCTTGACAGACCTCCAGCCGTACATGCGACAGTTCGTGGCTCACCTGCA  2400
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FIG. 5A

```
      2410       2420       2430       2440       2450       2460       2470       2080
GGAGACCAGCCCGCTGAGGGATGCCGTCGTCATCGAGCAGAGCTCCTCCCTGAATGAGGCCAGCAGTGGCCTCTTCGACG   2480
TCTTCCTACGCTTCATGTGCCACCACGCCGTGCGCATCAGGGGCAAGTCCTACGTCCAGTGCCAGGGGATCCCGCAGGGC   2560
TCCATCCTCTCCACGCTGCTCTGCAGCCTGTGCTACGGCGACATGGAGAACAAGCTGTTTGCGGGGATTCGGCGGGACGG   2640
GCTGCTCCTGCGTTTGGTGGATGATTTCTTGTTGGTGACACCTCACCTCACCCACGCGAAAACCTTCCTCAGGACCCTGG   2720
TCCGAGGTGTCCCTGAGTATGGCTGCGTGGTGAACTTGCGGAAGACAGTGGTGAACTTCCCTGTAGAAGACGAGGCCCTG   2800

      2810       2820       2830       2840       2850       2860       2870       2880
GGTGGCACGGCTTTTGTTCAGATGCCGGCCCACGGCCTATTCCCCTGGTGCGGCCTGCTGCTGGATACCCGGACCCTGGA   2880
GGTGCAGAGCGACTACTCCAGCTATGCCCGGACCTCCATCAGAGCCAGTCTCACCTTCAACCGCGGCTTCAAGGCTGGGA   2960
GGAACATGCGTCGCAAACTCTTTGGGGTCTTGCGGCTGAAGTGTCACAGCCTGTTTCTGGATTTGCAGGTGAACAGCCTC   3040
CAGACGGTGTGCACCAACATCTACAAGATCCTCCTGCTGCAGGCGTACAGGTTTCACGCATGTGTGCTGCAGCTCCCATT   3120
TCATCAGCAAGTTTGGAAGAACCCCACATTTTTCCTGCGCGTCATCTCTGACACGGCCTCCCTCTGCTACTCCATCCTGA   3200

      3210       3220       3230       3240       3250       3260       3270       3280
AAGCCAAGAACGCAGGGATGTCGCTGGGGGCCAAGGGCGCCGCCGGCCCTCTGCCCTCCGAGGCCGTGCAGTGGCTGTGC   3280
CACCAAGCATTCCTGCTCAAGCTGACTCGACACCGTGTCACCTACGTGCCACTCCTGGGGTCACTCAGGACAGCCCAGAC   3360
GCAGCTGAGTCGGAAGCTCCCGGGGACGACGCTGACTGCCCTGGAGGCCGCAGCCAACCCGGCACTGCCCTCAGACTTCA   3440
AGACCATCCTGGACTGATGGCCACCCGCCCACAGCCAGGCCGAGAGCAGACACCAGCAGCCCTGTCACGCCGGGCTCTAC   3520
GTCCCAGGGAGGGAGGGGCGGCCCACACCCAGGCCCGCACCGCTGGGAGTCTGAGGCCTGAGTGAGTGTTTGGCCGAGGC   3600

      3610       3620       3630       3640       3650       3660       3670       3680
CTGCATGTCCGGCTGAAGGCTGAGTGTCCGGCTGAGGCCTGAGCGAGTGTCCAGCCAAGGGCTGAGTGTCCAGCACACCT   3680
GCCGTCTTCACTTCCCCACAGGCTGGCGCTCGGCTCCACCCCAGGGCCAGCTTTTCCTCACCAGGAGCCCGGCTTCCACT   3760
CCCCACATAGGAATAGTCCATCCCCAGATTCGCCATTGTTCACCCCTCGCCCTGCCCTCCTTTGCCTTCCACCCCCACCA   3840
TCCAGGTGGAGACCCTGAGAAGGACCCTGGGAGCTCTGGGAATTTGGAGTGACCAAAGGTGTGCCCTGTACACAGGCGAG   3920
GACCCTGCACCTGGATGGGGGTCCCTGTGGGTCAAATTGGGGGGAGGTGCTGTGGGAGTAAAATACTGAATATATGAGTT   4000

      4010       4020       2030       4040       4050       4060       4070       4080
TTTCAGTTTTGAAAAAAAAAAAA 4023
```

FIG. 5B

MPRAPRCRAVRSLLRSHYREVLPLATFVRRLGPQGWRLVQRGDPAAFRALVAQCLVCVPWDARPPPAAPSFR
QVSCLKEL

VARVLQRLCERGAKNVLAFGFALLDGARGGPPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRRVGDDVLVH
LLARCALFV

LVAPSCAYQVCGPPLYQLGAATQARPPPHASGPRRRLGCERAWNHSVREAGVPLGLPAPGARRRGGSASRSL
PLPKRPRR

GAAPEPERTPVGQGSWAHPGRTRGPSDRGFCVVSPARPAEEATSLEGALSGTRHSHPSVGRQHHAGPPSTSRPP
RPWDTP

CPPVYAETKHFLYSSGDKEQLRPSFLLSSLRPSLTGARRLVETIFLGSRPWMPGTPRRLPRLPQRYWQMRPLFL
ELLGNH

AQCPYGVLLKTHCPLRAAVTPAAGVCAREKPQGSVAAPEEEDTDPRRLVQLLRQHSSPWQVYGFVRACLRRL
VPPGLWGS

RHNERRFLRNTKKFISLGKHAKLSLQELTWKMSVRGCAWLRRSPGVGCVPAAEHRLREEILAKFLHWLMSVY
VVELLRSF

FYVTETTFQKNRLFFYRKSVWSKLQSIGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRFIPKPDGLRPIVN
MDYVV

GARTFRREKRAERLTSRVKALFSVLNYERARRPGLLGASVLGLDDIHRAWRTFVLRVRAQDPPPELYFVKVD
VTGAYDTI

PQDRLTEVIASIIKPQNTYCVRRYAVVQKAAHGHVRKAFKSHVSTLTDLQPYMRQFVAHLQETSPLRDAVVIE
QSSSLNE

ASSGLFDVFLRFMCHHAVRIRGKSYVQCQGIPQGSILSTLLCSLCYGDMENKLFAGIRRDGLLLRLVDDFLLVT
PHLTHA

KTFLRTLVRGVPEYGCVVNLRKTVVNFPVEDEALGGTAFVQMPAHGLFPWCGLLLDTRTLEVQSDYSSYART
SIRASLTF

NRGFKAGRNMRRKLFGVLRLKCHSLFLDLQVNSLQTVCTNIYKILLLQAYRFHACVLQLPFHQQVWKNPTFF
LRVISDTA

SLCYSILKAKNAGMSLGAKGAAGPLPSEAVQWLCHQAFLLKLTRHRVTYVPLLGSLRTAQTQLSRKLPGTTLT
ALEAAAN
PALPSDFKTILD

FIG. 6

```
      10         20         30         40
GTCTCACTCTGTCACCCAGGCTGGAGTGTAATGGCACAAT    40
CTCGGCTCACTGCAACCTCTGCCTCCTCGGTTCAAGCAGT    80
TCTCATTCCTCAACCTCATGAGTAGCTGGGATTACAGGCG   120
CCCACCACCACGCCTGGTTAATTTTTGTATTTTTAGTAGA   160
GATAGGCTTTCACCATGTTGGCCAGGCTGGTCTCAAACTC   200

     210        220        230        240
CTGACCTCAAGTGATCTGCCCGCCTTGGCCTCCCACAGTG    240
CTGGGATTACAGGTGCAAGCCACCGTGCCCGGCATACCTT    280
GATCTTTTAAAATGAAGTCTGAAACATTGCTACCCTTGTC    320
CTGAGCAATAAGACCCTTAGTGTATTTTAGCTCTGGCCAC    360
CCCCCAGCCTGTGTGCTGTTTTCCTGCTGACTTAGTTCTA    400

     410        420        430        440
TCTCAGGCATCTTGACACCCCCACAAGCTAAGCATTATTA    440
ATATTGTTTTCCGTGTTGAGTGTTTCTGTAGCTTTGCCCC    480
CGCCCTGCTTTTCCTCCTTTGTTCCCCGTCTGTCTTCTGT    520
CTCAGGCCCGCCGTCTGGGGTCCCCTTCCTTGTCCTTTGC    560
GTGGTTCTTCTGTCTTGTTATTGCTGGTAAACCCCAGCTT    600

     610        620        630        640
TACCTGTGCTGGCCTCCATGGCATCTAGCGACGTCCGGGG    640
ACCTCTGCTTATGATGCACAGATGAAGATGTGGAGACTCA    680
CGAGGAGGGCGGTCATCTTGGCCCGTGAGTGTCTGGAGCA    720
CCACGTGGCCAGCGTTCCTTAGCCAGTGAGTGACAGCAAC    760
GTCCGCTCGGCCTGGGTTCAGCCTGGAAAACCCCAGGCAN    800

     810        820        830        840
GTCGGGGTCTGGTGGCTCCGCGGTGTCGAGTTTGAAATCG    840
CGCAAACCTGCGGTGTGGCGCCAGCTCTGACGGTGCTGCC    880
TGGCGGGGGAGNGTCTGCTTCCTCCCTTCTGCTTGGGAAC    920
CAGGACAAAGGATGAGGCTCCGAGCCGTTGTCGCCCAACA    960
GGAGCATGACGGGTTGGCTGTGTTCCGGCCGCAGAGCACC   1000

     1010       1020       1030       1040
GTCTGCGTGAGGAGATCCTGGCCAAGTTCCTGCACTGGCT    1040
GATGAGTGTGTACGTCGTCGAGCTGCTCAGGTCTTTCTTT    1080
TATGTCACGGAGACCACGTTTCAAAAGAACAGGCTCTTTT    1120
TCTACCGGAAGAGTGTCTGGAGCAAGTTGCAAAGCATTGG    1160
AATCAGACAGCACTTGAAGAGGGTGCAGCTGCGGGAGCTG    1200
```

FIG. 7A

```
      1210       1220       1230       1240
TCGGAAGCAGAGGTCAGGCAGCATCGGGAAGCCAGGCCCG   1240
CCCTGCTGACGTCCAGACTCCGCTTCATCCCCAAGCCTGA   1280
CGGGCTGCGGCCGATTGTGAACATGGACTACGTCGTGGGA   1320
GCCAGAACGTTCCGCAGAGAAAAGAGGGCCGAGCGTCTCA   1360
CCTCGAGGGTGAAGGCACTGTTCAGCGTGCTCAACTACGA   1400

      1410       1420       1430       1440
GCGGGCGCGGCGCCCCGGCCTCCTGGGCGCCTCTGTGCTG   1440
GGCCTGGACGATATCCACAGGGCCTGGCGCACCTTCGTGC   1480
TGCGTGTGCGGGCCCAGGACCCGCCGCCTGAGCTGTACTT   1520
TGTCAAGGTGGATGTGACGGGCGCGTACGACACCATCCCC   1560
CAGGACAGGCTCACGGAGGTCATCGCCAGCATCATCAAAC   1600

      1610       1620       1630       1640
CCCAGAACACGTACTGCGTGCGTCGGTATGCCGTGGTCCA   1640
GAAGGCCGCCCATGGGCACGTCCGCAAGGCCTTCAAGAGC   1680
CACGTCCTACGTCCAGTGCCAGGGGATCCCGCAGGGCTCC   1720
ATCCTCTCCACGCTGCTCTGCAGCCTGTGCTACGGCGACA   1760
TGGAGAACAAGCTGTTTGCGGGGATTCGGCGGGACGGGCT   1800

      1810       1820       1830       1840
GCTCCTGCGTTTGGTGGATGATTTCTTGTTGGTGACACCT   1840
CACCTCACCCACGCGAAAACCTTCCTCAGGACCCTGGTCC   1880
GAGGTGTCCCTGAGTATGGCTGCGTGGTGAACTTGCGGAA   1920
GACAGTGGTGAACTTCCCTGTAGAAGACGAGGCCCTGGGT   1960
GGCACGGCTTTTGTTCAGATGCCGGCCCACGGCCTATTCC   2000

      2010       2020       2030       2040
CCTGGTGCGGCCTGCTGCTGGATACCCGGACCCTGGAGGT   2040
GCAGAGCGACTACTCCAGCTATGCCCGGACCTCCATCAGA   2080
GCCAGTCTCACCTTCAACCGCGGCTTCAAGGCTGGGAGGA   2120
ACATGCGTCGCAAACTCTTTGGGGTCTTGCGGCTGAAGTG   2160
TCACAGCCTGTTTCTGGATTTGCAGGTGAACAGCCTCCAG   2200

      2210       2220       2230       2240
ACGGTGTGCACCAACATCTACAAGATCCTCCTGCTGCAGG   2240
CGTACAGGTTTCACGCATGTGTGCTGCAGCTCCCATTTCA   2280
TCAGCAAGTTTGGAAGAACCCCACATTTTTCCTGCGCGTC   2320
ATCTCTGACACGGCCTCCCTCTGCTACTCCATCCTGAAAG   2360
CCAAGAACGCAGCCGAAGAAAACATTTCTGTCGTGACTCC   2400

      2410       2420       2430       2440
TGCGGTGCTTGGGTCGGGACAGCCAGAGATGGAGCCACCC   2440
CGCAGACCGTCGGGTGTGGGCAGCTTTCCGGTGTCTCCTG   2480
GGAGGGGAGCTGGGCTGGGCCTGTGACTCCTCAGCCTCTG   2520
TTTTCCCCCAGGGATGTCGCTGGGGGCCAAGGGCGCCGCC   2560
GGCCCTCTGCCCTCCGAGGCCGTGCAGTGGCTGTGCCACC   2600
```

FIG. 7B

```
          2610       2620       2630       2640
AAGCATTCCTGCTCAAGCTGACTCGACACCGTGTCACCTA    2640
CGTGCCACTCCTGGGGTCACTCAGGACAGCCCAGACGCAG    2680
CTGAGTCGGAAGCTCCCGGGGACGACGCTGACTGCCCTGG    2720
AGGCCGCAGCCAACCCGGCACTGCCCTCAGACTTCAAGAC    2760
CATCCTGGACTGATGGCCACCCGCCCACAGCCAGGCCGAG    2800

          2810       2820       2830       2840
AGCAGACACCAGCAGCCCTGTCACGCCGGGCTCTACGTCC    2840
CAGGGAGGGAGGGGCGGCCCACACCCAGGCCCGCACCGCT    2880
GGGAGTCTGAGGCCTGAGTGAGTGTTTGGCCGAGGCCTGC    2920
ATGTCCGGCTGAAGGCTGAGTGTCCGGCTGAGGCCTGAGC    2960
GAGTGTCCAGCCAAGGGCTGAGTGTCCAGCACACCTGCCG    3000

          3010       3020       3030       3040
TCTTCACTTCCCCACAGGCTGGCGCTCGGCTCCACCCCAG    3040
GGCCAGCTTTTCCTCACCAGGAGCCCGGCTTCCACTCCCC    3080
ACATAGGAATAGTCCATCCCCAGATTCGCCATTGTTCACC    3120
CCTCGCCCTGCCCTCCTTTGCCTTCCACCCCCACCATCCA    3160
GGTGGAGACCCTGAGAAGGACCCTGGGAGCTCTGGGAATT    3200

          3210       3220       3230       3240
TGGAGTGACCAAAGGTGTGCCCTGTACACAGGCGAGGACC    3240
CTGCACCTGGATGGGGGTCCCTGTGGGTCAAATTGGGGGG    3280
AGGTGCTGTGGGAGTAAAATACTGAATATATGAGTTTTTC    3320
AGTTTTGAAAAAAAAAAAAGGAATTC 3346
```

FIG. 7C

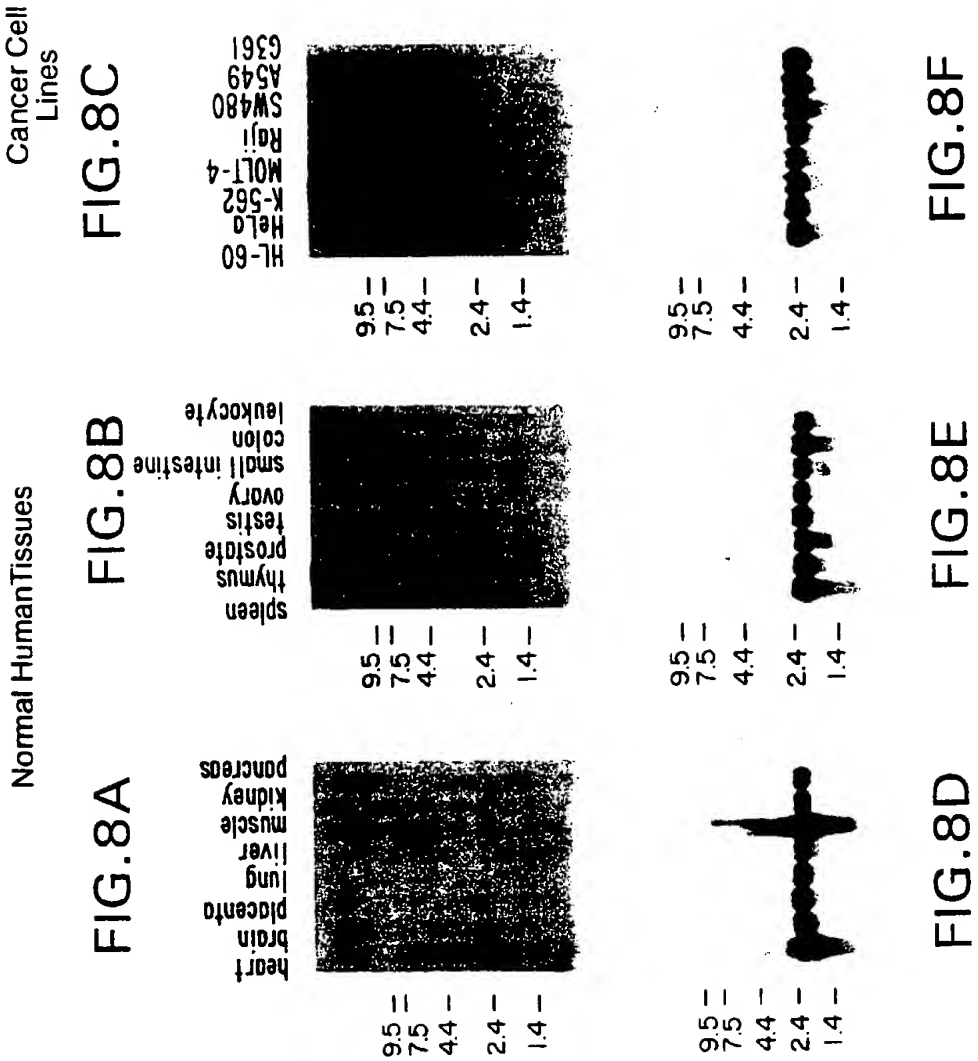

FIG.8A FIG.8B FIG.8C

FIG.8D FIG.8E FIG.8F

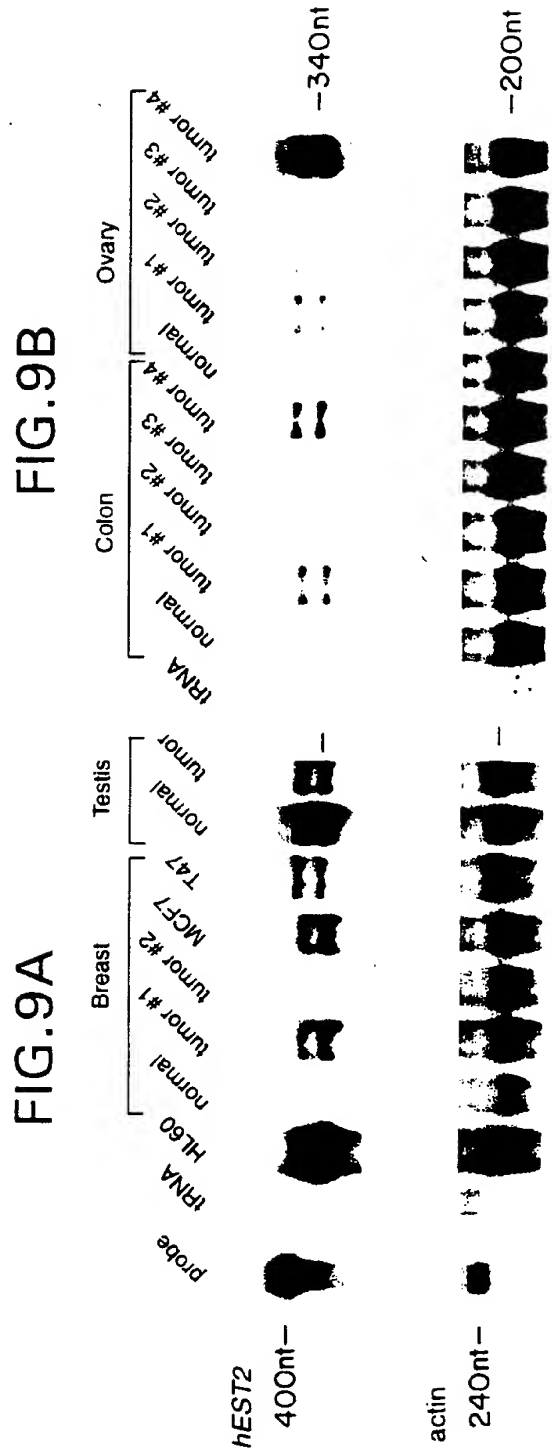

FIG.9A FIG.9B FIG.9C FIG.9D